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Preface

On behalf of the Sociedade Brasileira de Tecnologia Farmacêutica - SBTF and the organising committee, I welcome you to the *VI Pharmatech: III Annual Meeting of the Sociedade Brasileira de Tecnologia Farmacêutica* (SBTF)*, APGI Symposium on Membrane Transport and III Meeting of Pharmaceutical Quality Control (III ENECQ).* This event is part of a series of conferences entitled "Pharmatech", which have been held in Brazil since 1996. Pharmatech has established itself as one of the foremost Brazilian conference series in the pharmaceutical field. In the present edition, the SBTF joins the French *Association de Pharmacie Galénique Industrielle* - APGI in a partnership. As a result of it, for the first time this Conference assumes a true international character. Pharmatech 2001 seeks to continue the tradition of providing a leading-edge technical programme combined with an exciting parallel cultural programme. The main purpose of the Conference is to provide a forum for presentation and discussion of recent advances in pharmaceutical technology and pharmaceutical quality control, focused on both theoretical and practical aspects.

To celebrate this new century, the *VI Pharmatech* will begin by recapitulating revolutionary achievements in Pharmacy from Galen to the present. A special register should be addressed to the opening conference entitled Galenic Pharmacy: Its history and Evolution, written by Prof. Francis Puisieux_and Prof. Simon Benita, and presented by Prof. Benita. The scope of the Conference includes identification of both foreseeable and unforeseen challenges and problems that will impact the future of the pharmaceutical field. Among the topics that are gaining increasing importance, I would like to mention sustained and controlled release of biologically active agents, membrane transport, transdermal delivery systems, vaccine technology, and gene therapy; this listing being by no means exhaustive. Original approaches like nanotechnology unsettle apparent certainties and promise still quite unforeseeable developments. I would thus like this Conference to be illustrative of all these tendencies, without indeed claiming that its contents should be limited to the above topics. This conference has a marked interdisciplinary character that seems to me very timely since the application of new drug delivery systems tend not only to become more and more numerous, but also to move away from conventional drug administration. These Proceedings contain the text of the Plenary Lectures, Symposia Lectures, and 188 extended abstracts presented at the VI Pharmatech/APGI Symposium/III ENECQ held in Recife, Brazil, from August 5 to August 8, 2001. As always, the technical programme sessions are highlighted by invited lectures given by leading experts from academia and industry. The Programme presented in these proceedings was only possible due to the effort of many people, especially of the Scientific Committee members.

The Conference shall attract over 500 participants from Brazil and many other countries all over the world including France, England, Germany, Slovenia, Spain, Portugal, United States, Canada, Israel, China, Argentina, Uruguay, Chile, and Cuba. I hope this conference will provide many opportunities for the personal exchange of information and ideas.

I would like to thank the APGI for the successful partnership and for choosing Brazil to hold its bi-annual International Conference. I would like to mention my indebtedness to Amelie Bochot, General Secretary of the APGI, who has been a key person since the very beginning of the VI Pharmatech.

The organisers are deeply indebted to confirmed speakers who have agreed to contribute to the VI Pharmatech/APGI Symposium Membrane Transport/III ENECQ. I extend my thanks to all the authors who spent many hours on the preparation and correction of the submitted extended abstracts. I also would like to express my thanks to all three Committees, particularly to the Technical Programme Committee, for their valued work. Thanks and recognition are due, in addition, to many referees whose helpful scientific contributions have added credibility to these Proceedings. I wish to acknowledge the steadfast assistance of the young colleague Renato Sobral, who showed great competence in the editorial activities related to the preparation of these Proceedings. Last but no least, I wish to show my gratitude to the treasurer of VI Pharmatech, Adriana Carla Pontes de Oliveira, who was able to handle such a complex matter and who provided many a valuable advice. Finally I would like to express my deep gratitude to the various colleagues, members of the Conference Committee, who were involved in the Congress organisation and who contributed with enthusiasm, competence and efficiency, and in many cases with very hard work, to the success of this event. I truly appreciated the effort of them all. This event was made possible thanks to the financial support provided by Brazilian and French Government agencies for research, Government institutions and industries. I would like to express my gratitude to all financial contributors. Special thanks should be addressed to the Pernambuco Government agencies for their support to the cross-cultural programme.

Many thanks to everyone who helped to make the VI Pharmatech/APGI Symposium Membrane Transport and III ENECQ come true.

Nereide Magalhães Nereide Stela S. Magalhães *General Co-Chair, Brazil,* SBTF Assistant Secretary.

For the first time the French "Association de Pharmacie Galénique Industrielle" (APGI) and the Brazilian "Sociedade Brasileira de Tecnologia Farmacêutica" (SBTF) are honoured to invite you to participate in the *VI Pharmatech* **-** *International Conference on Pharmaceutics and Pharmaceutical Technology* which will take place in Recife in Brazil from 5 to 8 August 2001.This joint conference will be a living proof of the long collaboration between APGI and Brazilian scientists. Indeed, APGI is organising a Symposium day, devoted to **Membrane Transport** including the most recent advances in ophthalmic, oral absorption, prediction of absorption and others.

Prof. Dominique Duchêne, APGI President (1964-2000)
Dr. Jean-Yves Legendre, APGI President (2000-)

Honours and Awards

Honour Invited

Prof. **Francis Puisieux** will be honoured at the VI Pharmatech/APGI Symposium/III ENECQ in recognition of his contribution to the Pharmaceutical Profession in France and around the world, specially for supervising many Brazilian researches in the field of Pharmaceutical Technology.

Young Investigator Award

Cristália will sponsor the Young Researcher Award. Young investigators not holding a PhD degree and having the majority of their work carried out in one of the Brazilian Institutions.

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Sunday, August 5, 2001

Pre Congress Events

9:00 a.m.-5:00 p.m.	Local Registration
2:00 - 6:00 p.m.	Short Courses
	1. *Study and Application of Polymeric Coating* (Estudo e Aplicação de Revestimentos Poliméricos), Dr. Fernanda Raffin, UFRN
	2. *Pre-Formulation Studies* (Estudo de Pré-Formulação de Medicamentos), Dr. Newton L. Pereira, USP
	3. *Pharmacoeconomy* (Farmacoeconomia), Dr. Francisco Cavalcanti Júnior, CRF-SP
	4. *Design Strategies for Magistral Pharmacy* (Montagem Estratégica de Farmácia de Manipulação), Dr. Pedro Menegasso, CRF-SP

Congress Events

6:00 p.m.	Opening Ceremony
7:00 p.m.	Homage to Prof. Francis Puisieux
8:00 p.m.	**Opening Conference**: Galenic Pharmacy: Its History and Evolution Prof. Simon Benita, University of Jerusalem, Israel Prof. Francis Puisieux, University of Paris, France
9:00 p.m.	Welcome Reception

Monday, August 6, 2001

Symposium 1	Liposomes and Emulsion Technology
8:30 a.m.	Hydrophobically Modified Biomacromolecules. Relevance to Liposome Technology Dr. Adam Baszkin, University of Paris-Sud XI, France
9:10 a.m.	Liposomes as Tools for the Sustained Release of Bioactive Peptides in Specific Sites of the Brain Dr. Frédéric Frézard, University of Minas Gerais, Brazil
9:50 a.m.	Self Emulsifying Drug Delivery Systems for Improving Oral Absorption of Lipophilic Drugs Prof. Simon Benita, University of Jerusalem, Israel
10:30 a.m.	Symposia Discussion
10:45 a.m.	Coffee-break and poster viewing
Plenary Lecture	
11:30 a.m.	Advanced Pulmonary Formulation Systems – Prospects for Peptide, Protein & Systemic Drug Delivery Prof. John N. Staniforth and Dr. David Morton, University of Manchester, England
12:30 p.m.	Lunch
1:30 p.m.	*In vitro* Dissolution Studies Dr. Erick Beyssac, University of Laval, Canada
Symposium 2	Solid Dosage Forms
2:30 p.m.	Tableting of Coated Pellets into Disintegrating Tablets Prof. Peter-Christian Schmidt, University of Tübingen, Germany
3:10 p.m.	An Efficient Approach for Drying Polymeric Colloidal Systems Dr. Silvia S. Guterres, University of Rio Grande do Sul, Brazil
3:50 p.m.	Plasma Drug Level Calculation with Various Ways of Drug Delivery Prof. Jean-Maurice Vergnaud, University of St Etienne, France
4:30 p.m.	Symposia Discussion
4:45 p.m.	Coffee-break and poster viewing
Plenay Lecture	
5:30 p.m.	Non-invasive Methods for the Study of New Modes of Drug Delivery Prof. Jean-Marc Aiache, University of Clermond-Ferrand, France

Tuesday, August 7, 2001

APGI Symposium	**Membrane Transport**
6:30 p.m.	III Annual Meeting of the SBTF
6:30 p.m.	III ENECQ

Wednesday, August 8, 2001

Symposium 3	Toxicological Assays: Conventional and Alternative Methods
8:30 a.m.	General Aspects of Toxicological Assays Prof. Sérgio Luiz Dalmora, Federal University of Santa Maria, Brazil
9:10 a.m.	Cell Culture in Toxicological Assays Dr. Maria Pilar Vinardell Martinez-Hidalgo, University of Barcelona, Spain
9:50 a.m.	The alternative Toxicology. The Cell as one of the most Important Protagonist Dr. Gastón García Simón, LIORAD Laboratory, Cuba
10:30 a.m.	Symposia Discussion
10:45 a.m.	Coffee-break and poster viewing
Plenary Lecture	
11:30 a.m.	Recent Advances in Drug Analysis Prof. Fernando Mauro Lanças, University of São Paulo, Brazil
12:30 p.m.	Lunch
1:30 p.m.	Validity, Efficiency and Ethic in Biological Assays for Drug Development and Quality Control Dr. Adela Rosenkranz, University of Buenos Aires, Argentine
Symposium 4	Drug Delivery Systems
2:30 p.m.	Dendrimers - A Flexible Vehicle for Drug Delivery Dr. Antony D´Emanuele, University of Manchester, England
3:10 p.m.	Biodegradable Microspheres for DNA Vaccine Against Tuberculosis Dr. José M. Rodrigues Júnior, Federal University of Minas Gerais, Brazil
3:50 p.m.	Use of Food Protein as new Excipients for Bioadhesive and Sustained Release Drug Dosage Forms Dr. Erick Beyssac, University of Laval, Canada
4:30 p.m.	Symposia Discussion
4:45 p.m.	Coffee-break and poster viewing
Symposium 5	Pharmaceutical Quality Control
2:30 a.m.	Reference Standards Prof. Celso Figueiredo Bittencourt, Farmacopéia Brasileira, Brazil
3:10 a.m.	Sterilizing Processes Applied to Heat-Sensitive Materials Prof. Terezinha de Jesus Andreoli Pinto, University of São Paulo, Brazil
3:50 a.m.	Pharmaceutical Application of Thermal Analysis Prof. Rui Oliveira Macedo, Federal University of Paraíba, Brazil
4:30 p.m.	Symposia Discussion
4:45 p.m.	Coffee-break and poster viewing
Plenary lecture	
5:30 p.m.	Control of Processing and Formulation Variables to Achieve Desired Properties of Matrix Microspheres Dr. James C. Price, University of Georgia, Athens, USA
6:30 p.m.	Closing Ceremony and Folkloric show
7:30 p.m.	Ordinary Assembly of SBTF
9:30 p.m.	Gala Dinner

Poster Sessions

Monday, August 6, 2001: AM01-AM10; BD01-BD10; BI01-BI11; DD01-DD15; PP01-PP10; QC01-QC08
Tuesday, August 7, 2001: APGI01-APGI07; AM11-AM20; BD11-BD16; BI12-BI22; DD16-DD36; PP11-PP19
Wednesday, August 8, 2001: AM21-AM32; BE01-BD06; BI23-BI33; DD37-DD51; QC09-QC25

Francis Puisieux

Professor F. Puisieux graduated from Paris Faculty of Pharmacy in 1957. He received the C.E.S. (Pharmatechniques), the C.E.S. (Pharmacodynamiques) and the C.E.S. (Vegetable Physiology) in 1958 from the same Faculty. In 1959, he received the C.E.S. (Animal Physiology) and the C.E.S. (Organic Chemistry) from Sorbonne, Paris. The next year, he received the Doctorat degree (Docteur ès Sciences Physiques) from Sorbonne. He joined the staff of the National School of Medicine and Pharmacy, Caen (1962, Lecturer, 1964, *Maître de Conférences Agrégé de Pharmacie Galénique*). From 1968 to 1972 he joined the Paris Faculty of Pharmacy. Professor Puisieux was an Associate Professor from 1972 to 1974 at the Faculty of Pharmacy of Paris XI at Châtenay Malabry, and then Full Professor since 1974.

He was also appointed a "*Professor de Classe Exceptionale*" in 1981. In 1988, he spent two months as a Visiting Professor in Brazil. Prof. Puisieux hold a Visiting Professor position at the School of Pharmacy, University of Madison, USA, in 1988. He was also a Guest Professor in Pharmaceutical Technology at the School of Pharmacy, University of Jerusalem, Israel, in 1991. He was a Researcher Professor at the Government Industrial Research Institute, Osaka, Japan, 1992. He was also a Visiting Fellow at the Centre for Controlled Chemical Delivery at Salt Lake City, USA, in 1993. He served as the General Co-ordinator of the ERASMUS European Program, enrolling more than 30 students from 11 different European countries. His Research work was supported by a number of Industrial Contracts, as well as by Federal Grant, especially from the "*Centre National de la Recherche Scientifique*", CNRS. He also served as the Head (*Pharmacien Chef*) of the Central Pharmacy of the "*Hôpitaux de Paris*", responsible for new pharmaceutical formulas. Prof. Puisieux is a member of the National Academy of Pharmacy, France, a correspondent member of the Royal Academy of Medicine, Belgium, and is a member of the French Cosmetology Society. He is serving as a member of the French Society for Sciences and Pharmaceutical Techniques, a Fellow of "*Association Française des Enseignents de Pharmacie Galénique*" and the "*Group Thématique de Recherche sur les Vecteurs* (GTRV)". He has also been a member of the International Pharmaceutical Federation (FIP), a member of the Controlled Release Society (USA), and European Colloid and Interface Society (ECIS). He organised and chaired Conference sessions, and presented many overview lectures. He acts as an Expert of Board of Pharmaceutical Sciences of the FIP, and is also a Consulting-Expert for evaluation of Scientific Programs of the European Community (CEE) since 1988. Prof. Puisieux has been a Member of the National Commission for the French Pharmacopoeia, a member of the Central Council for the National Order of Pharmacists, France. He was appointed as a member of several official groups and working groups from the Ministry of Health, including the "*Commission d´Authorisation de Mise sur Marché des Médicaments*". He was Vice-president and then President of the Consultative Committee of French Universities. Professor Puisieux was awarded numerous distinctions. He won the Paris Faculty of Pharmacy award in 1958 and 1959, was awarded the 1961 Adrian Prize of the Chemistry Society of France and received the 1961 Moureu Medal. He is the recipient of the 1966 Nativelle Prize from the Medical Academy of France, and a 1979 Darolles Prize from the same academy. Prof. Puisieux also received an "*Palmes Académiques Chevalier*" in 1976 and he is the recipient of the 1989 **Order National du Mérite Chevalier**, France, and a 1990 Galien Prize. The 1998 Maurice-Marie Janot Lecture Prize, awarded jointly by APGI, the Paul Neumann Foundation for Scientific Research, and the *Association Francophone des Enseignants de Pharmacie Galénique* was bestowed to Professor Puisieux. He is an internationally recognised scientist and educator. He was the recipient of the *Doctorat Honoris Causa* from the Free University in Brussels, Belgium in 1997 and *the Doctorat Honoris Causa* from the Geneva University in 1998. **He will be honoured at the 2001 VI Pharmatech in recognition of his contribution to the Pharmaceutical Profession in France and around the world,** specially for supervising many Brazilian researches in the field of Pharmaceutical Technology. Professor Puisieux is a Member of the Editorial Board of the *Sciences et Technique Pharmaceutiques* (STP Pharma. Sci., *Editions de Santé*, Paris); Member of the Editorial Board of the J. Controlled Release; and a Member of the Editorial Board of the J. Drug Targeting. Professor Puisieux authored and co-authored 15 books and book chapters, and held 17 European patents. He supervised more than 30 Ph.D. Thesis and was responsible for more than 150 Ph.D. Students on his Laboratories. He has lectured widely as an invited speaker in over 18 countries at Universities in North America, Japan, European Countries and South America. He published 230 Journal articles, and more than 200 Proceedings or abstracts around the world.

OC GALENIC PHARMACY: ITS HISTORY AND EVOLUTION

S. Benita[1] and F. Puisieux[2]

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Galenic pharmacy, which in fact refers to dosage form formulation and drug compounding goes back to the most remote times of humanity. It was born the day one of our ancestors had the idea to modify the natural form of medication he was used to taking.

Our knowledge about the drug dosage forms is relatively recent. It goes back to the first writings about the art of medicine, which do not predate 3000 B.C.E. Among the oldest documents, the ones worth mentioning are the Pent Sao of China (2500 BC), the Babylonian Code of Hammurabi (2250 BC), the Vedas of India (around 2000 BC) and the Papyrus Edwin Smith and Ebers of Egypt (around 1500 BC).

In Antiquity the galenic forms were already very numerous and thanks to commercial ties, they were able to spread worldwide. In Greece and in Rome at the time of Galen for example, the dosage forms were practically the same as in China and in Egypt. In China, the medicines were, for the mostpart, infusions, decoctions, medicinal wines, electuaries, preserves and pills. The Egyptians used potions, herb teas, decoctions, macerations, mixtures, pills, boluses, lozenges, electuaries, and for the external use, poultries, ointments, eye lotions, plasters, creams, inhalations, fumigations, suppositories and enemas. In Greece, at the time of Hippocrates, the Greeks used baths, fomentations, fumigations, inhalations, decoctions, suppositories, enemas, pessaries, poultries, cerates, oils and ointments.

In the Middle Ages, the compounding of pharmaceuticals became separate from the practice of medicine. It was then that the first retail pharmacies appeared and the practice of Galenic pharmacy became officinal. The Renaissance and the following centuries did not bring tremendous changes in the art of drug preparation and compounding. The first Codex appeared in France in the 17th century, but the dosage forms remained almost the same.

The 19th century was rich in important milestone events for pharmacy in general and for galenic pharmacy specifically. These events include: the creation in France of the first schools of pharmacy and the individualization of the galenic pharmacy, the isolation of the pure active ingredients like morphine, quinine, strychine, digitalin, etc. In general this period was marked by a deep change in the world economy. Big enterprises were created in different domains such as metallurgy, textiles, colliery, chemistry, and pharmacy did not escape the changes. In the case of galenic pharmacy, the industrialization was favored by important inventions and marked innovations in the manufacturing methods. The major invention was without any doubt that of the pharmaceutical tablet patented by the Englishman Brockedon in the year 1843. In the U.S. and other Anglo-Saxon countries, the development of this new dosage form was fast and supported by the design of appropriate excipients and the construction of very efficient machines of production. It conquered a market share that was very important already before the end of the 19th century. In France, the tablet was not accepted right away in the beginning and its development was much slower. The invention of the soft capsule by Dublanc and Mothes in 1834 also played an important role in the development of the industrial production of the pharmaceutical dosage forms. Conceived by Lehuby in 1846, the hard gelatine capsule form had a rather late repercussion in France but gained a relatively important position in the U.S. since 1880.

Other innovations favoured the industrialization of the Galenic practice around the middle of the 19th century. Innovations worth mentioning are, for example, the effervescent tablet around 1880, the rotating tablet machine (1882), the invention of the ampoule (1886), and the development of the sterilization (research work of Pasteur).

Another major event favourable to the industrialization of the production of the galenic dosage forms was the implementation of the registered drug product. Instituted in France in 1941, it conferred to the industrially produced preparations a guarantee of quality and of safety that the compounding preparations produced in the retail pharmacy could barely pretend to reach.

Marked by the end of World War II, and by spectacular advances in science and technology, the last fifty years of the twentieth century was provided an opportunity for a real revolution in galenic pharmacy. Until the 1950s galenic pharmacy was confined to a simple packaging and presentation of the active ingredient. The development of the biopharmacy and pharmacokinetics beginning in the 1960's allowed it to attain a whole new dimension. Today galenic pharmacy has ceased to be just an art. It became a real science and now contributes its part in optimizing the fundamental qualities of every drug.

Galenic pharmacy became capable of contributing to the improvement of its bioavailability, to the control of the moment and place of its action, and to the improvement of the profit-risk ratio through more favorable distribution in the body and a better observance of the treatment. The progress in technology allowed, at the same time, for the modernization of pharmaceutical operations, the diversification of the excipients, the renewal of the modes of production and of packaging. For all these operations, the pharmacist now has available to him equipment that performs well and with a tremendous capacity as well as the ability to function in a continuous manner. New particulate techniques appeared around the 60's such as the microencapsulation, the microspherization, and in the 80's came the nanoencapsulation, the nanospherization, the spheronization-extrusion process, the spherical crystallization and the preeling. The excipients are now widely diversified and the semi-synthetic or synthetic products issued from the polymeric chemistry are numerous today. The packaging also improved immensely. For the solid forms, for example, the tubes, boxes or bottles of the 50's have given way to single packaging with identification of each unit.

In the last decade, ongoing efforts have been made to develop colloidal systems or drug carriers capable of delivering the active molecules specifically to the intended target organ while increasing the therapeutic efficacy by modifying the pharmacokinetic profile of various therapeutic classes of drugs.

There is still a lot to do however and galenic pharmacy does not lack challenges for the future. Tomorrow will probably bring an era of "E" pharmacy, and galenic pharmacy will have to be prepared to take up the challenge.

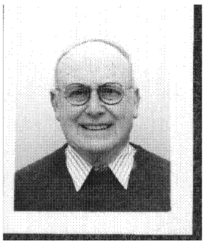
VI Pharmatech

III Annual Meeting of the SBTF

Jean-Marc Aiache

Professor Jean-Marc AIACHE was born on September 27, 1938 in Algiers (Algeria). He received his Master in Pharmacy in 1961 at the University of Algiers and, his Ph.D. in Clermont-Ferrand (France) in 1968. In 1972, he was appointed Professor in Clermont-Ferrand at the Faculty of Pharmacy and decided to create and thereafter, manage the first French Biopharmaceutics Department dedicated to the development of drug delivery systems, drug delivery devices, new "*in vitro*" drug release devices, correlations and/or relationships between the "*in vitro/in vivo*" data obtained from absorption or pharmacokinetics studies in volunteers. In this area, as a consultant and with the collaboration of pharmaceutical or cosmetic companies, he has approximatively developed 350 drug dosage forms or systems containing classical or well-known drugs or new chemical entities (peptides, hormones from biotechnologies...). These are presently marketed in France and/or in Europe (some of them came from his own patents). He is also the author or co-author of various books on Biopharmaceutics and Pharmacokinetics and of about 200 publications. He acts as an Expert in Pharmaceutical Technology and Biopharmaceutics and/or Analytical Chemistry for drug registration in Europe (pharmaceutical dossier). He is also a member of the French Commission of Pharmacopoeia, a Consulting-Expert member of Group 12 of the European Pharmacopoeia, a W.H.O. consulting expert for essential drugs and head of the W.H.O. Collaborating Centre for Biopharmaceutical Aspects of Drug Quality Control and past-President of the Academic Section of FIP (International Pharmaceutical Federation). Finally, he is also Associate Professor of many South American Faculties of Pharmacy (Uruguay, Argentina, Chile, Colombia, Brazil) and member of many Academies of Pharmaceutical Sciences. Prof. Aiache is a Member of 19 French and International Scientific Societies. The most important ones include: Society for Therapeutics and Pharmacodynamics, International Pharmaceutical Federation (FIP), American Pharmaceutical Association, American Academy of Pharmaceutical Sciences, International Society for Aerosols in Medicine, American Association of Pharmaceutical Scientists, American College of Clinical Pharmacology (status of fellow), Controlled Release Society. He received the following Scientific Distinctions**:** Prize of the French Cosmeceutics Society (1969), Two silver medals from the French Academy of Medicine (1974, 1976), and Pierre VELON Price awarded by the French Society of Cosmetology (1981). He also won the Academic Distinction**:** Decoration for services to education in France (Chevalier, 1992).

Adam Baszkin



Adam Baszkin is a Research Director at "*Centre National de la Recherche Scientifique*", and Head of the Physico-Chemistry of Surfaces Laboratory, University of Paris Sud. He received the Chemical Engineer M.Sc. (1957) from the Warsaw Technical University, and the Ph.D. in 1973 from the University of Paris, Sorbonne. In 1974, he joined the Physico-Chemistry of Surfaces and Membranes, CNRS, Paris. He was an International Visiting Scholar at the Biomedical Engineering Center, University of Utah, Salt Lake City (1978-1979). He was also appointed Visiting Research at Government Industrial Research Institute, Osaka, Japan, in 1988. He has presented Invited lectures at Universities in Japan, China, Brazil, Israel and various European countries (1988-2001).

Dr. Baszkin is a member of the American Chemical Society, the A.C.S. Division of Colloid and Interface Scientists and a member of the European Society of Biomaterials. He is the author of over 130 scientific publications, including journal papers and several book chapters. Dr. Baszkin supervised numerous graduate-level students in biophysicochemistry. He has been a member of the scientific committee of several international scientific meetings and workshops. His laboratory held many international agreements and collaborations, including: Laboratories of Biorelated Polymers, Artificial Cell Technologies, Kyoto University (Japan), Institute of Photographic Chemistry of Beijing (China), Federal University of Pernambuco (Brazil), The Hebrew University of Jerusalem (Israel), and Department of Physical Chemistry, University of Siena (Italy). Dr. Baszkin is currently a member of the editorial board of Colloid and Surfaces B: Biointerfaces, Elsevier Science Pub. B.V. His major research interests are in surface and polymer physical chemistry. They include development of quantitative methods of determination of functional groups at polymer surfaces, investigation on dynamic and structural properties of solid polymer films in response to changes in external environment and characterization of polymer-liquid interfaces and polymer-polymer self adhesive joins. Current research in focused on biointerfacial phenomena particularly applied to studies of surface interactions in biocomponent (proteins, enzymes) - polymer systems and to protein interactions with lipid monolayers in relation to molecular organization and recognition. His interest includes also self-assembly and properties of systems of low and high molecular weight amphiphilic compounds as well as drug-lipid and drug-polymer associations. Main experimental techniques in his laboratory include the use of radioactive elements (calcium 45, carbon 14) for adsorption studies of either inorganic ions or proteins on polymers, contact angle measuring devices for wettability determinations, surface tension, surface potential and surface pression measuring instruments for the characterization of monolayer of adsorbed and spread amphiphiles.

Simon Benita

Simon Benita received the B.Pharm. in 1972 and the Ph.D. degree in 1980, both from the Hebrew University of Jerusalem, Pharmacy Department. He joined the Hebrew University of Jerusalem (1978, Assistant; 1981 Lecturer; 1986 Senior Lecturer; 1991 Associate Professor), where he is a Full Professor since 1994. He was also Associate invited Professor at the Faculty of Pharmacy, University Paris-Sud (sabbatical) and served as Associate Guest Professor, Laboratoire de Pharmacie Galenique, University of Angers-France. Since 1975 he is Consultant to various local and international pharmaceutical companies. He has published 98 original journal papers, as well as 11 Review Articles. He holds 11 Patents and he has authored 87 Proceedings&Abstracts, and 12 Chapters. He is the Editor of two books: a) Microencapsulation: Methods and Industrial Applications, Marcel Dekker,N.Y. 1996 ; b) Submicron Emulsions in Drug Targeting and Delivery, Harwood Academic publishers, The Netherlands, 1998 and co-editor of Emulsions and Nanosuspensions for the Formulation of Poorly Soluble Drugs, eds., R. H. Muller, S. Benita and B. H. L. Bohm, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 1998. Prof. Benita research is focused on new microparticulate and nanoparticulate targetable drug delivery systems (microspheres, microcapsules, nanoparticles, nanocapsules, submicron emulsions, and self-emulsifying submicron emulsions) for either parenteral, ocular or oral administration. Advanced physical techniques are being used to elucidate the mechanism of formation of nanoparticles and the mechanism of stabilization of the submicron emulsions. Determination of drug release kinetics and release mechanism identification from all the above dosage forms are also performed. New colloidal and lipid delivery systems among which are the PLA nano- and microparticles, self-emulsifying oil formulations and the positively-charged submicron emulsions, are currently under investigation for drug targeting, enhanced oral bioavailability and controlled release of poorly absorbed drugs. As a result of his research activities, Prof. S. Benita formed and supervises a group of 21 M.Sc. students, 12 Ph.D. students and 8 post-doctoral students in Pharmaceutical Sciences. Prof. Benita is a Member of the Board of Pharmaceutical Sciences of the International Pharmacy Federation, Member of Editorial Board of Pharmaceutical Development and Technology; Member of Editorial Board of the Journal of Microencapsulation, Taylor & Francis, London; Member of Editorial Board of the S.T.P. Pharma Sciences, Editions de Santé (Paris)**;** Member of Editorial Board of the European Journal of Pharmaceutics & Biopharmaceutics; Governor of the International Microencapsulation Society**;** Governor of the Controlled Release Society (1995-1998); Member of International Advisory Board of Pharmaceutical Technology Conference.

Erick Beyssac

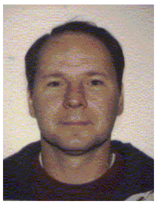
Erick Beyssac was born in 1962, France. He received the Master in Pharmacy, from the Faculty of Pharmacy, Clermont-Ferrand, France (1984) and the DESS in Biopharmacy, Faculty of Pharmacy, Biopharmaceutics Department, Prof. Aiache, Clermont-Ferrand, France (1985). He also received the Diploma in Statistics Applied to Biology, specialised in Clinical Researches (1988), from the University Pierre and Marie Curie, Paris VI and the Ph.D degree in Pharmaceutical Technology, from the Faculty of Pharmacy, Clermont-Ferrand, France (1989). He is an Associate Professor in Clermont-Ferrand (France) at the Biopharmaceutics Department under the responsibility of Pr. Aiache from 1985 until 2000.

He was in charge of the development of *in vitro* and biopharmaceuticals studies and responsible for the biopharmaceutical course for Pharm D students. Currently, he is an Invited Professor at the Faculty of Pharmacy of Université Laval in Quebec (from 2000 to 2002) and Co-director of the Research Group CIDAM, "Conception, Ingénierie et Développement de l'Aliment et du Médicament" (Conception, Engineering and Development of Food and Drug). Prof. Beyssac has authored more than 25 publications in various national and international journals. His research interests include the following: *In vitro / in vivo* correlations, Sustained drug dosage form development, and *In vitro* dissolution. He has over 40 communications as poster presentations, oral communications or invited conferences.

Antony D´Emanuele

Tony D´Emanuele is a senior lecturer in pharmaceutics at the School of Pharmacy and Pharmaceutical Sciences, University of Manchester. He graduated from the University of London in 1984 with a First Class honours degree in pharmacy. Whilst at London he received the ICI Award. He undertook his postgraduate studies at the University of Bath in the area of responsible electrophoretic drug delivery. He was awarded a Ph.D. in 1989 together with the Whittet Award. Dr. D´Emanuele took up his current appointment after an eighteen-month postdoctoral fellowship at the Massachusetts Institute of Technology where he investigated the use of ultrasound to modulate drug release from biodegradable implants. Dr D´Emanuele has been awarded two prizes whilst at Manchester, the Lilly Prize for Pharmaceutical Excellence (1995), and to the International Pharmacy Journal. Dr D´Emanuele has been a visiting scientist at the Department of Pharmacy, University of São Paulo and also the Department of Chemistry Engineering, Massachusetts Institute of Technology. Dr D´Emanuele has been invited to give over 50 lectures, and is the author of more than 100 publications. Dr D´Emanuele is a Member of the Royal Pharmaceutical Society of Great Britain, a Fellow of the Royal Society of Chemistry, and member of six other organisations. Dr D´Emanuele was the founder member of the United Kingdom chapter of the Controlled Release Society (1994) and in 1999 he was elected Chairman of the UK-Ireland Controlled Release Society. Dr D´Emanuele organised the 1997 UKICRS Conference in Manchester, a meeting that attract over 1000 participants and an international panel of speakers. It was the first pharmaceutical conference to be broadcast live on the Internet. Dr D´Emanuele´s research focuses on the delivery of bioactive molecules. His primary area of interest concerns the development of pulsatile and responsive drug delivery system. This work involves the novel application of polymers (including dendrimers and biodegradable polymers) to the development of delivery systems where release is responsible to a patient's varying requirements. A second major interest involves the delivery of genes into cells by means of sonication. His work on ultrasound delivery systems has result in the filing of two patents. Dr D´Emanuele led the initiative that brought the first environmental scanning electron microscope to a European university. In addition to his scientific interests, Dr D´Emanuele is also interested in information technology and was responsible for the creation of PharmWeb, the first structured pharmaceutical information resource on the Internet.

Frédéric Frézard



F. J. G. Frézard was born in Melun, France, in 1964. He received the Master degree in Molecular Biophysics in 1987 and the Ph.D. degree in Biophysics in 1990, both from the University of Paris VI (Pierre et Marie Curie). He was a Post-Doctorate Fellow at the University of Florida, USA, in 1991. From 1991 to 1994, he held a Visiting Research Position at the "*Centre National de la Recherche Scientifique*", CNRS, France. Then he joined the Fundação Ezequiel Dias, Brazil. In 1988, he joined the Department of Pharmacy at the Federal University of Minas Gerais, Brazil, where he is currently an Associate Professor. Dr. Frézard published about 25 journal papers and three book chapters (CRC Press, ACS Series, Polish Scientific Publishers). He has authored more than 50 Conference papers (Proceedings articles and abstracts). His main interest areas are in Biophysics of Process and Systems, polymer and colloids, Human Protozoology and Immunology. He is a Reviewer of Biochimica et Biophysica Acta, and Cell Biology International.

Jean-Maurice Vergnaud

J.M. Vergnaud was born in 1932, France. He received the Ph.D. degree in Catalysis (1960) and the Ph.D. in Gas Phase Chromatography (1965). He is currently a Professor at the University of St Etienne, France. Prof. Vergnaud has published more than 420 papers, and presented over than 250 Communications and Conferences. He supervised 70 thesis and he is a member of the Board of Editorial Advisory of five International Journals (J. of Polymer Engineering, London; Drying Technology, USA; Polymer Testing, U.K.; Polymer and Polymer Composites, U.K.; Synthetic Polymer Journal, Bucharest etc.). Professor Vergnaud is the author of four books: Liquid Transport Processes in Polymeric Materials. Modelling and Industrial Applications, Prentice-Hall, NY, 1991; Drying of Polymeric and Solid Materials. Modelling and Industrial Applications, Springer-Verlag, London, 1992; Cure of Thermosetting Resins: Modelling and Experiments, Springer-Verlag, London, 1992; Dosage Forms for Controlled Release with a Polymer Matrix, E. Horwood ed., London, 1993. He was also a former Director of a group of Laboratories and Vice-President of the University of St. Etienne. He was the recipient of a number of awards, including The French Industrial Society Award for new methods in Gas Phase Chromatography (1965); A Silver Medal by the French Academy of Sciences for research on molecular sieves (1977) and George S. WHITBY Prize by the Rubber Division, ACS (1998). His main research interest includes the Processes in Polymer by considering the reaction and heat transferred through the polymer, and modelling of the process; The study of Processes in Polymers by considering the matter transferred through the polymer and reaction and; The Study of Processes of matter transferred between polymer packages and food, with or without functional barrier, as well as attempts for food safety. He has been working on vulcanisation of elastomers, cure of thermosets and thermoset coatings, Process in Calorimetry and Fire Retardant Coating, Plasticised PVC coatings, Dosage forms for Controlled Release, Drug transfer in the plasma and tissues, various transports in Polymers and solid materials.

James Clarence Price

J.C. Price received the B.S. (Pharmacy) from the University of Arkansas in 1953, and the M.S. (Pharmacy) from the University of Utah in 1957. In 1963, he received his Ph.D. degree from the University of Rhode Island. He has been working in the area of Pharmaceutics, especially Physical and Chemical aspects of Pharmacy, Controlled Release, Microencapsulation, Tablet Technology and Formulations. He is currently a full Professor of Pharmaceutics, College of Pharmacy, University of Georgia and an Associate Professor Pharmaceutics, College of Pharmacy, University of Rhode Island. He participated to a number of special training programs, especially FDA training Courses. Prof. Price has published 66 journal papers in wide-range areas of Pharmacy, supervised 32 thesis, and presented about 75 communications in Conferences around the world. He organized and chaired numerous Conference sessions, workshops, and presented overview lectures. Prof. Price is also involved on various grant, and he was the Principal Investigator of more than 20 Research Projects, most of them supported by EMC Foundation and National Cancer Institute. Dr. Price is a member of the American Pharmaceutical Association, The Academy of Pharmaceutical Sciences, The American Association of Pharmaceutical Scientists, and The Controlled Release Society. His current research interests are in Controlled Release Floating Microspheres, Zero-Order-Release Matrix Microspheres and Site Specific Oral Dosage Forms.

Peter Christian Schmidt

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PL-01 HYDROPHOBICALLY MODIFIED BIOMACROMOLECULES. RELEVANCE TO LIPOSOME TECHNOLOGY

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Chemical modification of proteins, enzymes and polysaccharides by covalent attachment to their molecules of hydrophobic moieties yields surfactant-like molecules with extremely interesting properties.

Cholesterol or fatty acid-conjugated biomarcomolecules display :

(i) enhanced emulsifying and foaming activities, (ii) spontaneously form colloidal clusters in aqueous media and (iii) form organised monomolecular films at the air/water interface.

The preserved specific recognition of these hydrophobically modified macromolecules enables their unique application in (i) mimicking of structures and functions of various supramolecular assemblies of biomembranes, (ii) drug targeting and immunodiagnostics both based on emulsions, nanoparticles (clusters) and liposomes and (iii) biosensor membrane preparations with improved molecular architecture.

Information on the molecular structure of systems formed from hydrophobically modified molecules may be obtained from monolayer studies assessed from the measurements of surface potential and surface pressure of such monolayers.

We report here surface properties of a series of cholesteryl-pullulan derivatives (CHP) and of a modified glucose oxidase GO(mod) obtained by grafting to its lysine residues alkyl C16 chains. For CHP molecules we will show how it is possible to quantify the number of penetrating into egg-phosphatidylcholine monolayers cholesteryl anchors and for GO(mod) we report the influence of the lipid chain length and of polar headgroup charges upon the efficiency and effectiveness of GO(mod) penetration into lipid monolayers.

For CHP molecules, we will demonstrate that they are capable of penetrating egg-phosphatidylcholine monolayers even at high surface coverages and that both the steric repulsion exerted by polysaccharide chains extended towards the aqueous environment and the uncompensated lipid potential explain why CHP coated liposomes are stable for long periods of time.

The reported data are based on the following published papers :

[1] B. DEME, V. ROSILIO & A. BASZKIN
Polysaccharides at Interfaces I. Adsorption of Cholesteryl-Pullulan Derivatives at the Solution-Air Interface. Kinetic Study by Surface Tension Measurements.
Colloids and Surfaces B : Biointerfaces 4, 357-365 (1995)

[2] B. DEME, V. ROSILIO & A. BASZKIN
Polysaccharides at Interfaces II. Surface Potential of Adsorbed Cholesteryl-Pullulan Monolayers at the Solution-Air Interface
Colloids and Interfaces B: Biointerfaces, 4, 367-373 (1995).

[3] A. BASZKIN, M.M. BOISSONNADE, V. ROSILIO, A. KAMYSHNY & S. MAGDASSI
Adsorption of Hydrophobized Glucose Oxidase at the Solution/Air Interface
J. Colloid Interface Sci., 190, 313-317, (1997)

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Penetration of Glucose Oxidase and of the Hydrophobically Modified Enzyme into Phospholipid and Cholesterol Monolayers
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PL-02 LIPOSOMES AS TOOLS FOR THE SUSTAINED RELEASE OF BIOACTIVE PEPTIDES IN SPECIFIC SITES OF THE BRAIN

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Introduction

Several features of liposomes make them particularly suitable for the sustained release of bioactive (poly)peptides *in vivo*. Liposomes offer a “natural” environment that usually preserves their structure and biological activity. Furthermore, liposomes can protect encapsulated (poly)peptides from enzymatic degradation and rapid *in vivo* clearance. Liposomal immunomodulators, mainly endogenous cytokines and exogenous peptides from bacterial origins, have been extensively studied [1]. In contrast, endogenous vasoactive peptides received little attention, despite their involvement in several important chronic-degenerative diseases, mainly arterial hypertension and diabetes.

This presentation will focus on the work of our group with the vasoactive peptides of the Renin-Angiotensin System (RAS) and, more specifically, on the application of liposome technology for the study of the physio-pharmacological actions of these peptides. The potential biomedical applications of these formulations will also be discussed.

RAS is an hormonal circulating system involved in the regulation of blood pressure and salt and fluid homeostasis. Furthermore, it is an autocrine/paracrine modulator of tissue functions (heart, blood vessels, kidney, brain and endocrine glands) [2]. The most important effector products of RAS are the octapeptide Angiotensin II (Ang II) and the amino terminal fragment angiotensin-(1-7) (Ang-(1-7)). Figure 1 documents the pathways for the endogenous production of both peptides, as well as some of their specific biological actions.

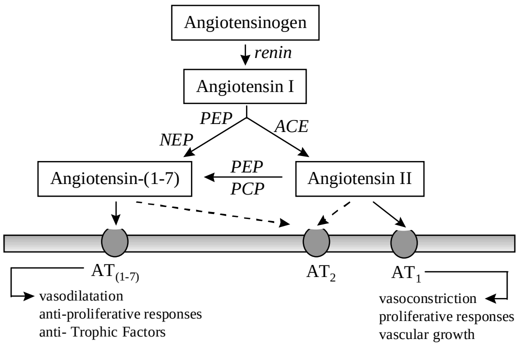

Figure 1. Simplified illustration of the proteolytic pathways for the formation of Ang II and Ang-(1-7). Ang-(1-7) and Ang II recognize angiotensin receptor subtypes to convey appropriate differential responses. ACE, angiotensin converting enzyme; PEP, prolyl-endopeptidase; PCP, prolyl-carboxypeptidase; NEP, neutral-endopeptidase.

Methods

The physio-pharmacological importance of these pathways is illustrated by the fact that they offer targets for two important families of anti-hypertensive drugs: the Angiotensin-Enzyme converting inhibitors and the AT_1-receptor antagonists.

One of the main interests of our group is the role of RAS peptides in the central control of blood pressure. The methodology conventionally employed in these investigations consists of a single microinjection of the peptide (or its antagonist) in a specific site of the brain. As a major drawback, this methodology fails to mimic the chronic conditions of the peptide endogenous production, mainly because of the very short half life of RAS peptides. This context prompted us to evaluate the potential of liposomes for the sustained release of RAS peptides in specific sites of the brain. Ang-(1-7) was selected for this study because of its known pressor effect at the level of the rostral ventrolateral medulla (RVLM) and because of the considerable interests in its long-term physio-pharmacological effects [3].

Results

Ang-(1-7) was encapsulated in different types of liposomes (differing in size, membrane fluidity and surface properties) and the resulting preparations were micro-injected unilaterally in the RVLM of Wistar rats. The effects on blood pressure (MAP) and heart rate (HR) were registered by telemetry. Among the different liposome preparations tested, only sterically-stabilized liposomes made from high-melting phospholipid (190 nm average diameter) were able to produce long-lasting alterations of MAP and HR. More specifically, this preparation elicited a significant pressor effect on day-time and bradycardia on night-time that lasted for 5 and 3 days, respectively [4]. These cardiovascular effects resulted in a significant attenuation of the circadian variations of MAP and HR. None of these effects was observed following micro-injection of empty liposomes. From the physiological point of view, this study established, in chronic conditions, the pressor effect of Ang-(1-7) at the RVLM. Moreover, it unmasked a new physiological role for Ang-(1-7) at the level of the RVLM: modulation of the circadian rhythms of MAP and HR. From the methodological point of view, this novel approach represents a significant advance and should contribute to the elucidation of the physiopatological role of RAS peptides in the brain.

Conclusion

Our study also established that the bioavailability of RAS peptides is markedly enhanced upon liposome encapsulation. Given the biological importance of these peptides, especially their ability to regulate tissue growth and blood pressure, several therapeutic applications of these liposome formulations can be envisioned. For instance, the ability of Ang-(1-7) to inhibit angiogenesis and tissue growth [3] could be explored for the treatment of angiogenesis-dependent diseases, such as cancer and diabetes complications. On the other hand, the angiogenic and proliferative activities of Ang II may found application in the induction of wound healing and post-ischemic tissue revascularization.

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PL-03 SELF-EMULSIFYING DRUG DELIVERY SYSTEMS FOR IMPROVING ORAL ABSORPTION OF LIPOPHILIC DRUGS

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Introduction

Self-emulsifying drug delivery systems (SEDDS), which are isotropic mixtures of oils and surfactants, are considered a promising and preferred approach to improve oral availability of lipophilic drugs. Self-emulsifying formulations spread readily in the GI tract, while the digestive motility of the stomach and intestine provides the agitation necessary for self-emulsification. For lipophilic drugs which display dissolution rate-limited absorption, these self-emulsifying lipid formulations may offer improvement in the rate and extent of absorption, while yielding more reproducible blood-time profiles and resulting in significant improvement of the drug bioavailability [1].

The aim of this work was to evaluate the effect of SEDDS on the oral bioavailability of lipophilic drugs, such as cyclosporine A (CsA). In addittion, the influence of droplet size and charge of resulting emulsions on drug uptake by intestinal mucosa and oral bioavailability was examined.

Experimental section

- **Composition of SEDDS**

Only very specific combinations of pharmaceutical excipients lead to efficient self-emulsifying systems [2]. Relatively high surfactant concentrations (usually more than 30% w/w) are needed in order to produce an effective self-emulsifying system. The excipients include surfactants such as polyglycolyzed mono-, di- and triglycerides, polyglycolyzed glycerides and Tween 80, oils such as ethyl oleate (EO), olive oil, medium-chain saturated fatty acids, hydrolyzed corn oil, and solvents such as ethanol, propylene glycol, PEG 400, glycerol and benzyl alcohol (BA). The positively charged SEDDS include cationic lipids such as oleylamine (OA) and stearylamine.

- **Preparation of SEDDS**

The preparation of SEDDS consisted of accurately weighing and mixing various components until a clear solution is obtained, which spontaneously results in either a microemulsion or an emulsion upon dilution with aqueous solutions. Triangular phase diagrams with different excipient combinations were performed using CsA as the model drug in an attempt to identify the different coarse and colloidal dispersion zone areas (the data will be presented). Positively charged emulsions were prepared by adding about 3% OA in the SEDDS, while negatively charged formulations were prepared without OA. The exact compositions are shown in Table 1.

- **SEDDS Evaluation**

The droplet size of resulting emulsions was determined by the photon correlation spectroscopy method (PCS). The Zeta potential of resultant emulsions was measured by electrophoresis utilizing Malvern Zetasizer 3000 (Malvern, UK).

- **Oral bioavailability studies**

Positively and negatively charged medicated emulsions for *in vivo* use were prepared by SEDDS aqueous dilution just prior to the administration. Blood CsA levels were determined by RIA, using ImmuChem™ Coated tube kit (ICN Biomedicals, Inc., Costa Mesa,California, USA).

Results and Discussion

It was shown that SEDDS may represent an attractive alternative to orally administered emulsions, since they are physically stable lipid solutions incorporated in standard soft gelatin capsules [1].

The effect of lipids on the bioavailability of orally administered drugs is highly complex due to numerous mechanisms by which lipids can alter the biopharmaceutical characteristics of the drug. They include: decreased rate of gastric emptying, increased dissolution rate of the drug and solubility in the intestinal fluid, and formation of lipoproteins promoting lymphatic transport of highly lipophilic drugs. Factors such as acid chain length of triglyceride, saturation degree and volume of lipid administered may affect drug absorption profile and its blood/lymph distribution.

Table 1: Formulations of self-emulsifying drug delivery systems.

Excipients	Composition percentage of excipients			
	(⁻) charged*	(+) charged*	(⁻) charged**	(+) charged**
CsA	5	5	5	5
Tween	25	25		
Ethanol	30	30		
Oleylamine	0	3	0	3
Ethyl oleate to	100	100		
Labrafil 2609			25	25
Arachis oil to			100	100

*Droplet size was about 200-500 nm following 1:50 dilution of SEDDS with water. **Droplet size was a few microns following 1:50 dilution of SEDDS with water.

Oil emulsion droplets entering the GI tract undergo structure changes. They may be broken and restructured. However, mixed micelles and microemulsions can penetrate through the aqueous layer and the mucin and are absorbed by one of the following ways: pinocytosis, diffusion or endocytosis. The active drug will reach the systemic blood circulation via the portal vein or through the lymphatic system.

The droplet size/bioavailability correlation is much more complex in positively charged emulsions as compared to negatively charged emulsions. This was investigated in a Sprague Dawley rat oral bioavailability study, where groups of six rats were given different emulsion formulations of CsA resulting from SEDDS dilutions (Table 1). The results of oral absorption of CsA in different emulsion formulations resulting from SEDDS dilutions as a function of droplet size and surface charge are presented in Figure 1. It is shown that the CsA blood levels of positively charged SEDDS were significantly higher than those of negatively charged (without OA) formulation. However, no difference in blood level profiles was noted as a function of droplet surface charge when the mean droplet size of the emulsions resulting from SEDDS dilution (Tween 80, ethyl alcohol/ethyl oleate with and without OA) was in the range of 200-500 nm. The relative bioavailability of positively charged coarse emulsions calculated using the linear trapezoidal rule was 8.0 times higher than that of the same but negatively charged coarse emulsions (AUC_{0-24} of 652 ng·h/ml and 72.6 ng·h/ml, respectively), while both positively and negatively charged fine emulsions were not significantly different (AUC_{0-24} of 947.4 ng·h/ml and 1112.8 ng·h/ml, respectively). For negatively charged emulsions, the relative bioavailability of the fine emulsions was 14.3 times higher than that of coarse emulsions. For positively charged emulsions, however, the relative bioavailability of the fine emulsions was only 0.5 times higher than that of coarse emulsions. The above results suggest the dominant role of the dissolution rate improvement in the CsA absorption caused by droplet size reduction. It can be deduced that although positively charged emulsions with larger oil droplets may help to overcome charge neutralization by negatively charged mucus, the negatively charged emulsions with smaller droplet size is more efficient in enhancing the oral bioavailability of cyclosporine A. On the other hand, the positively charged formulation may be recommended in dosage forms containing micron size particles destined for oral use.

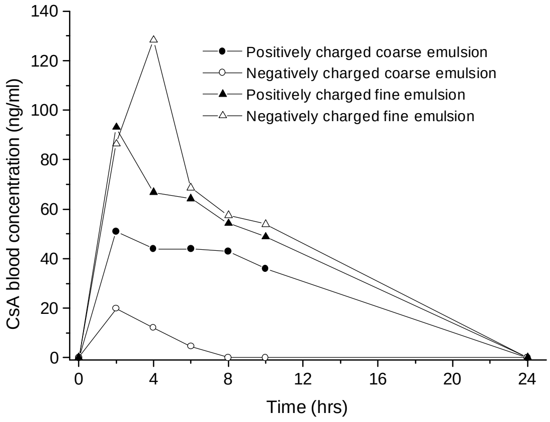

Fig. 1: CsA blood levels in rats as a function of droplet size and surface charge of emulsions resulting from 1:50 dilution of different SEDDS with water. Coarse emulsions (the mean droplet size, a few microns) composition (%w/w): Labrafil 2609 25/OA 0 or 3/arachis oil to 100. Fine emulsions (the mean droplet size, 200-500 nm) composition (%w/w): Tween 80 25/ ethyl alcohol 30/OA 0 or 3/ethyl oleate to 100.

Conclusions

SEDDS are versatile and offer numerous possibilities for development of suitable dosage forms to improve bioavailability of poorly absorbed lipophilic drugs. However, the correlation between bioavailability and physicochemical properties, such as droplet size and zeta potential, is very complex. Therefore, many biological aspects of these formulations are still unclear and merit further investigation.

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PL-04 ADVANCED PULMONARY FORMULATION SYSTEMS PROSPECTS FOR PEPTIDE, PROTEIN & SYSTEMIC DRUG DELIVERY

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Efficient pulmonary delivery highly desirable but not easily achieved. Direct administration of b-2 agonists and steroids, which revolutionised the treatment of asthma, used a range of very inefficient systems, delivering a variable and small dose fraction to the target site. It is now recognised that na improvement in both the efficiency and the reproducibility of delivery can widen the application, giving either improved local treatment, or more significantly, systemic activity.

Systemic delivery applies particularly to administration of proteins and peptides. But where the ability to invent new molecules has outrun our capacity to deliver them by routes other than injection, there are other simpler molecules which could benefit from a rapid onset of action which follows alveolar deposition, reduced first-pass or other metabolism and chemical degradation in the gastro-intestinal environment.

Many now regard achieving reproducible and efficient deep lung penetration of suitable doses demands a dry powder inhaler starting point, and requires an exquisite balance between the design of drug, of 'inactive' particle surfaces and of the device dynamics. A great deal of attention has been given to device engineering, providing more efficient metering systems and airways turbulence. Similary, much attention has focussed on creating drug particles with the most appropriate aerodynamic size and shape, for example using supercritical fluid processing. Left at this point, neither ultrafine drug particles, nor efficient devices provide an adequately functional aerosol system.

It is now becoming evident that further performance improvements can be made through advanced formulation design. Such improvements have resulted in new physico-chemical entities, providing the formulator with greater control of inter-particle forces, and a better marriage between the powder and dispersion demands of the device hardware. For example, formulations are enhanced by the modulation of microscopic surface domains at critical drug & carrier contacts. This can be realised by the use of nanophase Force Control Agents, combined into the formulation using appropriate production methods. The macroscopic re-suspension characteristics of the bulk powders can be enhanced by judicious design of particle powder agglomerate. Together, using a formulation-integrated dry powder inhaler product development approach, these improvements create a viable opportunity to deliver systemically acting drugs via the pulmonary route.

A secondary, but increasingly important, requirement to achieving reproducibly efficient alveolar penetration is to modulate drug absorption. Formulation improvements which can be applied to achieve these aims include those which allow control of the rate of drug diffusion to and across the pulmonary mucosa; bionhanced absorption; protection form recognition by the RES following absorption.

Pulmonary delivery of drugs is now a major opportunity created by integrated application of particle engineered drug, carrier and force control agents with high efficiency active dry powder inhalers and any bio-modulating formulation agents. Formulation integrated dry powder inhalers offer the prospect of improving dose reproducibility and penetration, with the potential for reducing dose size and frequency, in a patient-friendly delivery system.

PL-05 COMPRESSION OF COATED PELLETS INTO DISINTEGRATING TABLETS

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Pellets as elements of multi unit dosage forms show several biopharmaceutical advantages compared to monolithic ones. They are transported from the stomach into the intestine comparable to liquids and independent from the presence of food [Clarke et al. 1993, 1995], the intra- and interindividual variations of plasma levels are reduced and the total bioavailability could be enhanced [May and Rambeck 1989, Hosny et al. 1998]. Pellets are normally administered to the patient as hard gelatin capsules due to the simple filling process in pharmaceutical production. Nevertheless capsules are more expensive compared to tablets and they are nondividable. Therefore an increasing interest in tablets containing pellets is observed during the last few years. Their production is cheaper and they could be divided into subunits [Beckert 1995, Beckert et al. 1996]. On the other hand there are some risks in the production of pellet containing tablets:

- demixing processes in the tableting mixture
- pellet damages during the tableting process
- changes in disintegration and dissolution time of the active ingredient after tableting causing a change in bioavailability.

Pellets having sustained release or enteric coating properties could be either of the matrix or the coated type. Tableting matrix type pellets is in general less critical compared to coated ones. Within the group of coated pellets enteric coatings need much more care in processing than sustained release films because any enteric coating has to comply with the requirements of USP 24 for enteric coated articles indicating that within two hours in 0.1 M hydrochloric acid the drug dissolution is limited to 10 % in maximum.

Factors influencing the tableting behaviour of pellets are:

- the nature of the pellets themselves, whether they are brittle or plastically deforming which depends on the excipients and methods used for their production,
- the size of the pellets: small pellets are less sensitive to demixing processes on a tableting machine,
- the properties of the coating, which must be flexible enough to withstand the forces of the tablet press. In addition pellets brought into contact during compression should not stick together enabling the tablet to disintegrate,
- the thickness of the coating

Bisacodyl, a laxative, being marketed as an enteric coated dosage form, was chosen as a model drug. It was sprayed onto placebo pellets of a diameter between 0.85 and 1.00 mm from an aqueous suspension in a fluidized bed coater using a small amount of Eudragit L 30 D as a binder. The pellets were then enteric coated using either an Eudragit L 30 D or an Eudragit FS 30 suspension, the latter being much more elastic. Thus a very sensitive model with respect to pellet damages was achieved. Both pellet types fulfilled the requirements of USP 24 with respect to enteric coated articles liberating less than 10 % of bisacodyl in 0.1 M hydrochloric acid during two hours. With Eudragit L 30 D it was not possible to develop pellet containing tablet formulations complying to USP 24. Neither an exchange of tablet excipients nor the adition of different plasticizers and the doubling of the coating thickness could overcome the brittle nature of this film forming agent. The addition of Eudragit NE 30 D to L 30 D films reduced the film damages due to the high elasticity of NE 30 D but increased at the same time the disintegration and the dissolution time in artificial intestinal fluid.

The new polymer Eudragit FS 30 D comprises of 10 % methacrylic acid, 25 % methyl methacrylate and 65 % methyl acrylate. It is soluble above pH 7.2, the glass transition temperature is 48 °C without the addition of a plasticizer, the minimum film forming temperature was determined to 14 °C (Lehmann 1995). The elongation at brake in the presence of 5 % and 10 % triethyl citrate is around 50 and 300 % respectively showing the high elasticity of the film. As an antiadhesive the use of 8% glyceryl monostearate is recommended(Petereit et al. 1995). Using 10 % triethyl citrate as a plasticizer an increase of sticking tendency of the pellets could be observed (Wesseling et al. 1999). Therefore in the studies presented here either 0.5 % of Aerosil 200 and 2.5 % of talc were admixed to the pellets.

The pellet distribution within a tablet using coloured pellets was investigated in dependence of the speed of the rotary press (Korsch Pharma 230) and the particle size of the fill/binder. Coarse granules of the filler/binder led to a high degree of pellet separation at higher machine speeds, while fine ones showed no segregation tendency. A more detailed investigation of the pellet damages showed that mainly pellets at the tablet surface are destroyed during tableting while pellets inside the tablet remained intact. Pellet damages are directly correlated to the surface of the tablet. A ball shaped tablet should show the lowest percentage of damaged pellets. The optimized formulation contains 60 % of bisacodyl pellets, coated with 25 % dry substance of Eudragit FS 30 D, 10 % triethyl citrate (based on the content of Eudragit FS 30 D), 29.5 % Avicel PH 101 and 0.5 % magnesium stearate. These tablets showed a hardness between 100 and 130 N, a disintegration time of 9 to 10 min, a friability of less than 0.1 % and a bisacodyl release in 0.1 M hydrochloric acid during the first two hours of 4.3 to 6.8 %. Thus the preparation complies with the requirements of USP 24 regarding enteric coated articles.

PL-06 AN EFFICIENT APPROACH FOR DRYING POLYMERIC COLLOIDAL SYSTEMS

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Colloidal polymeric suspensions such as nanocapsules and nanospheres have been developed as drug targeting delivery systems. Besides the i.v. route, colloidal systems have also been administered orally, either for systemic uptake or local contact within gastrointestinal tract. Besides the i.v. route, colloidal systems have also been administered orally, either for systemic uptake or local contact within gastrointestinal tract. For peroral administration, the nanoparticles could be administered in the form of an aqueous dispersion as the final dosage form. However these systems present a physico-chemical instability essentially due to the particles sedimentation or/and poor stability of drugs in aqueous environment. Suspensions of indomethacin-loaded nanocapsules are stable by seven months [1], suspensions of diclofenac-loaded nanocapsules, by eight months [2], and suspensions of indomethacin-loaded nanospheres are stable for six months [3]. This is an important technological problem, which can compromise the industrial feasibility of such systems. Therefore, it is necessary to improve the stability of these forms to reach a shelf-life of several years.

During the past few years, studies have been devoted to developing solid forms of polymeric colloidal systems. Although reports in the literature show that freeze-drying is a good method to dry nanospheres [4]. Freeze-drying was also used to dry nanocapsules containing indomethacin prepared with poly[(±)-lactic acid] and benzyl benzoate [5]. However, after the redispersion in water of freeze-dried nanocapsules stored at room temperature, losses of drug even as large as 50 % were observed. Gürsoy and coworkers [6] attributed these results to changes in capsule wall structure and Chasteigner and coworkers [5] pointed out that during the freezing step, water crystallization stress can break the nanocapsules, promoting the leakage of their contents into the continuous phase.

The spray-drying technique exhibit advantages like low price, easiness of industrial transposition and possibility of preparation of powders with better established physico-chemical characteristics than freeze-drying powders. Recently, we reported the first application of this technique to dry nanocapsules suspensions with the view to obtain a solid form of polymeric nanocapsules containing anti-inflammatory drugs such as diclofenac and indomethacin [7,8].

Gastrointestinal side-effects such as irritation, ulceration and mucosal damage can occur after administration of non-steroidal anti-inflammatory drugs [9]. A variety of mechanisms including a direct "barrier breaking" effect, due to the local contact between the mucosæ and solid drug particles, and an indirect effect, due to the local and/or systemic inhibition of enzymatic systems capable of producing protective substances, such as PGE_2 and prostacyclin, may cause these side-effects. The effectiveness of colloidal drug delivery systems (liposomes and nanospheres or nanocapsules suspensions) to reduce potential side-effect, such as gastrointestinal ulceration in the case of oral administration of anti-inflammatory drugs, was evaluated in several investigations.

This communication describes the application of spray-dried technique to dry suspensions of diclofenac and indomethacin-loaded nanocapsules and nanospheres prepared by the interfacial precipitation method. The suitability of different components of formulations for preparing powders as well as the physico-chemical characteristics of the powders obtained is discuss. For the preparation of colloidal suspensions different polymers were assayed: poly(ε-caprolactone), poly[(±)-lactic acid] or Eudragit S90®; and for the nanocapsules formulations benzyl benzoate or Mygliol 810® as oily phase. Additionally the aim of the present study was to verify the potential of spray-dried polymeric nanocapsules and nanospheres to improve the gastrointestinal tolerance of diclofenac after repeated intragastric administration. To verify the absorption of the drug from the spray-dried powders reconstituted in water, their plasma concentration time profiles were determined in rats after oral administration in a pharmacokinetic investigation.

It was demonstrated that without drying adjuvant, it was not possible to spray-dry the nanocapsules and nanospheres suspensions, due to the strong adhesion of the product onto the spray-dryer walls. This problem was avoided by addition of colloidal silicon dioxide (3.0 % w/v), which is widely reported as a spray-drying adjuvant. The spray drying yield for the powders prepared from colloidal suspensions was between 50-70% showing a low moisture content for all cases.

The scanning electron microscopy analysis shows that the spray-dried powders are spherical particles, characteristic that usually lead to a free-flow and represent an important characteristic for the application of spray-dried powders as an intermediary pharmaceutical product. However, in this case, the most interesting characteristics observed were related to the spray-dried microparticles surface. While the microparticles of silicon dioxide obtained from the suspensions usually show a rugged surface, with the presence of some cavities, the microparticles prepared from a mixture silicon dioxide and nanocapsules (prepared using Mygliol 810® as oily phase) or nanospheres colloidal suspensions do not show the presence of cavities and it was clearly observed the nanoparticles adsorbed on the surface of silicon dioxide. The micrographs of the surface of spray-dried powders surface showed the silicon dioxide microparticles completely covered by nanostructures of about 300 nm for nanocapsules formulations and 70 nm for nanospheres formulations [8,10].

On the other hand, when benzyl benzoate was employed in the composition of nanocapsules suspensions, the morphologic study showed that after spray-drying it was not possible to visualize the presence of adsorbed structures around 200 nm onto the surface of silicon dioxide at a magnification of 25,000x, despite the rugged surface. These results can be explained by the solubility of those polymers in the benzyl benzoate oil phase. Despite various publications using benzyl benzoate as oil phase in the preparation of nanocapsules suspensions, the present study clearly showed that this ester is not appropriate to formulate nanocapsules, due to the solubility of PLA and PεC in it. In brief, it was demonstrated the influence of the oil phase in the formulation of nanocapsules suspensions prepared from pre-formed polymers. To assure that the oil phase is a non-solvent for the polymer, a simple swelling experiment can be carried out [10].

The stability study showed that diclofenac or indomethacin-loaded nanocapsules and nanospheres suspensions are poorly stable. Applying spray-drying technique it was possible to obtain powders from diclofenac or indomethacin-loaded poly(ε-caprolactone) nanocapsules or nanospheres which presented stable drug recovery and morphological characteristics after five months of storage at room temperature.

The diclofenac-loaded nanocapsules suspension and the corresponding spray-dried nanocapsules induced a marked protective effect on gastrointestinal tissues as compared to the ulcerative effect observed with the diclofenac sodium salt solution. Similar protection was previously observed for diclofenac-loaded nanocapsules colloidal suspension which was attributed to the avoidance of the direct contact of the drug with the gut wall [11].

One could argue that the protective effect observed for spray-dried diclofenac nanocapsules formulation could be associated with a lack of absorption of drug through the gastrointestinal tract. In order to refute this hypothesis, the plasma profiles of the drug were analyzed after oral administration of the spray-dried diclofenac nanocapsules and aqueous solution of diclofenac sodium salt in equivalent single dose. The diclofenac sodium salt solution presented a peak concentration of 4.75 ± 0.22 µg/mL at 30 min, with an elimination half life of 7.5 h. These parameters were similar to those reported in the literature for this drug [11]. A plasma concentration rebound was observed after 2 hours of drug administration. This result could be explained by the enterohepatic circulation reported for diclofenac after oral administration in rats [12]. Although this hypothesis is consistent, it should be consider, in this work, that the second peak could also be attributed to the averaging of the data points from different animals to obtain the mean plasma profile, as described by Kendall *et al*. [13]. In this way, the second peak could be artificial, resulting from averaging different T_{max} of the individual profiles. The AUC_{0-t} was determined as 406 µg*min/mL. For spray-dried diclofenac nanocapsules a different plasma profile was obtained. The peak concentration (1.91 ± 0.41 µg/mL), obtained at 15 min, was significantly lower than the observed for the aqueous drug solution. A concentration rebound was not observed for this formulation and its elimination half-life was 9.3 hours. This half-life suggests that diclofenac from spray-dried nanocapsules probably presents enterohepatic circulation as reported for the solution of the drug [12]. The AUC_{0-t} was determined to be 435 µg*min/mL, very similar to the AUC determined for the solution. These results show that although diclofenac presented a different profile after the administration of diclofenac sodium salt solution or spray-dried nanocapsules, it was completely absorbed from the formulations. The relative bioavailability determined was 1.07. Other authors already reported an increased relative bioavailability when comparing the non-steroidal anti-inflammatory drugs in aqueous solution with the drug associated in nanocapsules [14].

In conclusion, spray-drying technique can be an interesting approach to dry nanocapsules or nanospheres suspensions and to obtain nanocapsules incorporated in a solid form [7,8,10]. Diclofenac-loaded spray-dried nanocapsules is valuable in reducing the gastrointestinal irritant effect of this drug[15].

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PL-07 PLASMA DRUG LEVEL CALCULATION WITH VARIOUS WAYS OF DRUG DELIVERY

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Introduction

Amongst the various ways of drug delivery, the orally taken dosage forms with controlled release are widely known. The drug is dispersed through a polymer in order to control the dissolution rate of the drug along the gastrointestinal tract. Thus the main drawbacks of the conventional dosage forms, e.g. a fast release that is responsible for high peaks alternating with low troughs of the drug in the plasma, high dosage frequency, bad therapy and possibility of non-compliance, are considerably reduced. Two extreme types of polymers are used for the matrix. Depending on the nature of the polymer, the process of drug release as determined from *in vitro* test is controlled either by diffusion with stable polymer or by erosion with erodible polymers [1]. One main problem in therapy is to determine the plasma drug level, obtained through *in vivo* test.

Thus the Food and Drug Administration (FDA) managed two workshops in order to correlate the plasma drug level with the kinetics of drug release (*in vitro/ in vivo* correlation) but the problem was found to be unrealistic [2].

A new method was developed for calculating the plasma drug level by taking into account the kinetics of drug release (*in vitro* test) and the pharmacokinetic parameters [3], by using numerical models. Thus they made possible the evaluation of the characteristics of the dosage forms (nature of the polymer, shape, dimensions) necessary for the desired therapy [4].

Moreover, by extending these numerical models, the drug level in various tissues such as blister fluid [5], the lung and bronchial secretion has also been calculated [6].

Theoretical

- **Kinetics of the drug release by erosion**

With a constant rate of erosion, the radius of a dosage form, spherical in shape, decreases linearly with time. And thus the amount of drug release after time t as a fraction of the corresponding amount initially in the dosage form is expressed in terms of time t and time of full release t, [1]:

$$\frac{M_t}{M_{in}} = 1 - \left(1 - \frac{1}{t_r}\right)^3 . \qquad (1)$$

- **Kinetics of the drugs release by diffusion**

With a constant diffusivity D and a very high coefficient of convection on the surface [6], the kinetics of the drug release is expressed in terms of a time for a sphere of radius R by the series [1]:

$$\frac{M_\infty - M_t}{M_\infty} = \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp\left(- \frac{n^2 \pi^2 D t}{R^2} \right), \qquad (2)$$

where M_t and M_∞ are the amount of the drug released after time t and infinite time, respectively.

- **Calculation of the plasma drug level**

Whatever the process of drug release, the rate of the drug amount X in the gastrointestine, can be obtained by

$$\frac{dX}{dt} = F_t - k_a X_t , \qquad (3)$$

where F_t is the rate of drug release by the dosage form. The rate of the drug amount Y in the plasma is given by:

$$\frac{dY}{dt} = k_a X_t - k_e Y_t , \qquad (4)$$

where k_a e k_e are the rate constants of absorption and elimination.

As the mathematical treatment is not feasible when F_t is not constant or linear with time, the problem is resolved by building numerical models taking all the facts into account, e.g., the stages of drugs release along de G.I. tract time, the stages of absorption into and elimination out of the plasma.

- **Calculation of the drug in various tissues**

The amount of drug transferred in the blister fluid is calculated by considering the diffusion of the drug from the plasma into the with a constant, especially that there is a low permanent of the drug through the skin,

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2}. \qquad (5)$$

The amount of drug transferred into the lung tissue is calculated by considering the diffusion of the drug through the lung, as described by eq. 5 with the boundary condition:

$$C_{lung} = K'C_{plasma}, \qquad (6)$$

meaning that at the lung-plasma interface, the drug concentration is in the lung K' times that in the plasma [6]. The amount of drug transferred into the bronchial secretion is calculated by using the eq. 1-6, with in addition the following at the lung-sputum interface

$$-D\frac{\partial C}{\partial x} = h\left(C_{lung,t} - K_{sputum,t}\right), \qquad (7)$$

where D is the diffusivity of the drug in the lung, h is a convection factor into the sputum depending on its viscosity, and K is a partition factor at this interface meaning that there is a drop in the drug concentration from the lung to the sputum. Finally, these numerical models make possible the calculation of the drug level in various sites: the GI, the serum, and various tissues.

Results and discussion

The validity of the models has been tested in various cases: plasma, blister fluid, lung tissue and mucus. Two types of results are considered: those concerned with the dosage forms and the nature of the polymer, the others with the drug level in the plasma and the tissues. The polymer matrix plays the major role for the rate of drug release. The stable polymer with the process of diffusion is responsible for the following facts a high rate of drug release at the beginning of the process, this rate decreases exponentially with time meaning that all the drug initially in the dosage form is released after infinite time [4]. The effect of the GI tract time is of importance, and it is a limitation for the intake frequency of the dosage forms. The erodible polymer with the erosion process is of great interest. The rate of drug release in the G.I. or measured through *in vitro* test is nearly constant from the beginning to the end of the process, all the drug is released out of the dosage form at a given time t_f.

As a result, more constant plasma drug levels are obtained with erodible polymer. Moreover, this type of polymer can be used without harm for adhesive dosage forms, enabling the time of full drug release to exceed largely the GI tract time. This fact is of great importance in various cases, and especially in noncompliance (intake omission, or double dosing), since the drug concentration in the plasma and in other tissues such as the lung or even the mucus is not dramatically varied [6].

Conclusion

These theoretical studies made by building numerical models taking all the facts into account, e.g., the kinetics of drug release and the pharmacokinetic parameters, have shown that these calculated results are in good agreement with the experimental results obtained in various laboratories. It is thus possible to predict the drug level in the plasma and in other tissues, when these data are previously determined. Moreover the characteristics of the dosage forms, such as the nature of the polymer matrix, the dimension and shape, can be evaluated in order to meet the requirements of the desired therapy, leading to shorter times of study for developing new dosage forms.

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PL-08 NON INVASIVE TECHNIQUES FOR THE STUDY OF NEW MODES OF DRUG DELIVERY

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Rational For These Techniques

Many studies have been directed towards an understanding of the manufacturing processes and properties of solid oral dosage forms. Despite this, the development of such products is essentially empirical, although increasingly guided by the results of such studies.

This empiricism is, however, required as the majority of solid pharmaceutical systems are, of necessity, complex. The results of scientific studies on simple, well-defined systems, cannot therefore always be directly applied in practice. In addition, there is a lack of understanding of the behaviour of solid pharmaceuticals after administration.

Furthermore, it is well Known that bioavailability of a drug from a formulation is influenced by a complex interplay of physiological and physiochemical factors. However, the primary determinant of absorption is the rate at which the drug is resealed from the formulation into solution which in turn depends on the rate of disintegration, for example, of tablets or the properties of the excipients used that increase the amount of drug exposed to the medium. The drug must be dissolved in the human fluids (GI or the fluids) to be absorbed and hence the absorption of many drugs especially-those with poor water solubility, is dissolution rate limited.

The ability to control the rate of presentation of a drug and achieve a desired in vivo behaviour, by manipulation development and generated the need for in vitro tests, which would allow the effects of manufacturing variables to the studied. The knowledge that the pH of body fluids changed-along the gastrointestinal tract from stomach to colon increased the need for sophistication of the tests and attempts were made to begin to simulate in-vivo conditions. The majority of drugs are weak acids or bases and the dissolution is therefore dependent upon the pH of the gut fluid. There is considerable variation in the pH within the gastrointestinal tract, and most physiology texts state that the gastric pH is in the range - 3, with a pH of 5 - 6in the duodenum, increasing to 7-8 in the proximal jejenum and 8 in the large intestine. However, there is some evidence that the pH of the fasting stomach in man may be much higher.

The muscular actions of the gastrointestinal tract stir and agitate the preparation during its transit, thus a paddle was incorporated into the dissolution apparatus to break -up the stagnant diffusion layers of fluid. Levy found that agitation tablets in the stomach as observed by x-rays was mild and his observations were used to, decide stirring conditions for in vitro dissolution tests. Further refinements include conducting the test at body temperature and the addition of digestive enzymes and surfactants such as pepsin, bile salts an lecithins, since these have been shown to affected *in vitro* dissolution.

The ability to determine drug in body fluids enabled researchers to examine the effect to formulation variables on bioavailability. It soon became clear that the application of simple in vitro tests was inadequate to explain the behaviour of some preparations.

The dissolution of a dose form and the release of a drug in some instances does not correlate with the absorption of the drug into systemic circulation. The application of various designs of in vitro apparatus to stimulate absorption was largely unsuccessful and investigators turned to other methods of trying to explain the relationship between the release characteristics of a formulation and the plasma concentration -time profile. In addition, demand arose for more sophisticated formulations especially sustained or controlled release to fulfil three aims:

-Firstly, there was an increasing number of observations that certain drugs were irregularly absorbed from the gastrointestinal tract. This led to the concept of absorption windows, in which the intestinal contents or nature of the epithelium of specific areas of the gastrointestinal tract optimised absorption, and it became important to the pharmacist to take advantage of this phenomenon to increase bioavailability.
-Secondly, there was increasing attention paid to the application of enteric coatings and release products to avoid local toxicity.
- Thirdly, there was an attempt to improvise patient compliance in multiple daily dosing regimens and the reduction of the minimisation of peaks and troughs in the plasma concentration time profile. This led to the development of news systems which attempted to reduce the number of daily doses of a drug, releasing the drug slowly within the gastrointestinal tract over a period of hours. These sustained release preparations can be formulated either as single or multiple unit dose forms. A major concern with sustained release devices is that since they contain up to a whole day's dose of drug in a single unit, they may dose-dump with serious consequences for the patient.
Actually, we are receiving new drugs coming from Biotechnology and it is also necessary to study

routes of administration other than the oral route, for example, nasal, ocular, vaginal, rectal, pulmonary, routes.

The visualisation of the behaviour of the dosage form and the demonstration of the drug absorption from these routes is an important research goal to aid in the development of new technology systems.

Study of Drug Dosage Forms

1- General considerations

Solid oral dosage forms generally swallowed whole with a glass of water. The dosage form goes through the oesophaghs into the stomach where it will remain for a period of time. During this time it may disintegrate and dissolve to a greater or lesser extent the residence time stomach and the amount of dissolution occurring will be important as drugs must be solution to be absorbed and the major site of absorption is the small intestine. The absorption of a given drug may not, however, occurs equally along the length of the small intestine. Hence, the rate through the intestine will also be important.

2- Techniques

Many techniques have been proposed, some are direct other are imaging ones. We will not mention the direct ones because they are obsolete but better focus on the imaging methods.

The first one was the X - ray technique developed in 1933, but now, the most important of them is the gamma scintigraphy

Definition and area of interest

Gamma scintigraphy allows the passage of the formulation throughout the gastrointestinal tract to be monitored and stasis of a formulation can easily be detected. The position of a formulation and the degree of dispersion within the gastrointestinal tract can be related to the simultaneous plasma concentration of the drug. Simultaneous pharmacokinetic and seintigraphic profiles for a formulation have facilitated the design of suitable dosage forms for drug with poor bioavailability.

The gamma camera has a large field of view, which can be split up into the equivalent of a matrix of several thousand finely collimated gamma detectors. Gamma camera imaging can be carried out using two alternative methods, static imaging in which single acquisitions are stored, and dynamic imaging in which a sequence of data of varying frame time can be obtained. The latter techniques is used to follow rapid processes, such as drainage of an aqueous formulation from the eye. Normally, gastrointestinal transit is sufficiently slow to be resolved by static imaging, however, dynamic imaging is required to study oesophageal transit.

An important advantage of this technique is that the field of view can be arbitrarily divided up into areas and the amount of isotope within these areas can be accurately quantified, and hence the movement and distribution can be followed.

A limitation of the technique of gamma scintigraphy is that very little anatomical information is gained, unless the formulation outlines easily recognised organs such as the stomach and large bowel,. When non- disintegrating matrix systems are studied. Identification of the position of the object becomes difficult and it is necessary to administer a second radio-pharmaceutical to outline the gastrointestinal tract.

Related techniques

It is to be noted that a number of techniques are related to gamma scintigraphy but they require different instrumentation.

- **Tomography**

This technique has been used to study the deposition of aerosols; the total lung space can be seen and if the image of the distribution of the aerosol is superimposed, the efficiency of lung deposition can be assessed.

- **Positron emission Tomography**

Is differs fundamentally form the techniques described so far and involves the detection of gamma rays emitted by positron emitters.

- **Perturbed angular correlation spectroscopy**

It is not an imaging method can usefully be performed concurrently with an imaging study. The technique is based on the 2 gamma rays decays cascade of indium-111.

- **Applications**

Drug dosage form

To date, gamma scintigraphy has been used to investigate the behaviour of a wide variety of dosage forms including tablets, capsules, suspensions, multiparticulates, aerosols, rectal foams, suppositories, osmotic pumps and ocular inserts.

New Methods

- **Magnetic Resonance Imaging (MRI)**

Visualisation of the digestive tract may be improved by the application of oral contrast media. To our knowledge, only a few MRI studies on in vivo localisation of dosage forms have been carried out in humans and animals.

The last but not the least.

Magnetic Source Imaging Method

It is possible to continuously monitor the gastrointestinal transit of magnetically marked, solid, oral dosage forms with multi-channel bio-magnetic measuring equipment and by magnetic source imaging (MSI) methods. For the investigations presented, a sucrose, pellet was coated with powdered magnetite (Fe3o4) in polymethyl methacrylate.

References can be asked to the authors.

PL-09 DENDRIMERS - A FLEXIBLE VEHICLE FOR DRUG DELIVERY

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Dendrimers are well-defined macromolecules that have a specific size, shape and chemical functionality. Structurally they are highly branched macromolecules that can be subdivided into three architectural components: a central core branched cell, interior branch cells, and branch cells possessing surface groups. They are synthesised through a stepwise repetitive reaction sequence [1], which gives rise to different generations of the same molecule and determines the size and surface functionality of the macromolecule. The surface groups of dendrimers are amenable to modification and can be tailored for specific applications

It has been demonstrated that dendrimers can enhance the solubility of low aqueous solubility drugs [2] either by inclusion of the drug in the dendritic architecture or through surface interaction via either electrostatic or covalent linkage. It has also been shown that dendrimers may be used as a delivery system for the controlled release of bioactive materials [3,4]. In addition, the ability of Starburst® dendrimers to function as potential carriers for the delivery of genetic material [5] and anticancer agents has been demonstrated [6]. It was suggested that the polycationic surface of the dendrimer macromolecule may interact electrostatically with biologically relevant anions, such as nucleic acids.
Starburst® polyamidoamine (PAMAM) dendrimers are a specific family of dendritic polymers, which are based on an ammonia or ethylene diamine core and an amidoamine repeat branching structure [7]. These dendrimers can be synthesised in a variety of well-defined molecular weights. Their size and surface functionality is defined by the number of controlled repetitive additions of monomer units giving rise to different generations. The effect of surface modification e.g. PEGylation [4] on the properties of Starburst® dendrimers has been examined.

The presentation will consider the unique properties of dendrimers and provide an overview of the pharmaceutical applications of these versatile polymers.

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PL-10 POLYMERIC CARRIERS FOR DNA VACCINE AGAINST TUBERCULOSIS

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Introduction:

Immunization procedures that present the antigen to the immune system as an endogenous antigen should be an interesting strategy, especially when the development of a cellular immune response is required. In this respect, DNA vaccines have shown exciting results in several experimental models of infectious diseases. Concerning DNA vaccination, the levels of protein expression after direct injection are transient due to the eventual loss of non-integrated plasmid DNA from transfected nuclei. Many strategies have been proposed for improving the DNA stability and many gene vectors are nowadays in trials. Viral vectors and liposomes have been widely proposed but they still fail if considering the main requirements for vaccine design such as safety, stability and/or immune response modulation. The gene gun technique, which consists of adsorbing the plasmid onto gold microparticles and shooting the host's dermis, has shown excellent results in terms of expression efficiency with very low DNA amounts. However, as in the intramuscular injection method, boosters are still necessary and the problems related with non-biodegradable support remain to be solved. Indeed, this technique seems to modulate specifically a TH2 immune response, which is not interesting for protection of many infection diseases. Thus, a sustained delivery of plasmid at the tissue may represent a source of continuous gene uptake thereby providing the maintenance of expression. One promising strategy proposed to solve these problems is the use of polymeric delivery systems, which are polymers that release their content in a controlled manner continuously over time.

Among the polymeric devices currently used, biodegradable microspheres such as those based on poly-lactic poly-glycolic co-polymers (PLGA) have the potential to act as mediators of DNA transfection targeted to phagocytic cells such as macrophages, and to protect it against biological degradation by nucleases. PLGA is a biocompatible and biodegradable material with an extensive record of safe use in medicine. Microspheres uptake by antigen-presenting cells has been extensively studied and is directly related to particle diameter. Microspheres less than 10 μm in diameter are actively taken up by APC cells such as macrophages, and those smaller than 5 μm in diameter are taken up by the M cells of the Payer's patches of the small intestine, being of special interest in the design of oral vaccines. The ability of these systems to elicit the immune response at mucosal surfaces constitutes an additional advantage especially against pathogens that gain access through mucosal surfaces such as the respiratory and gastrointestinal tracts.

As far as the development of new strategies for vaccination against tuberculosis is concerned, the vaccination by intramuscular injection of naked plasmid DNA encoding mycobacterial 65-kDa heat shock protein (hsp65) protected BALB/c mice against subsequent challenge with virulent *Mycobacterium tuberculosis* H37Rv strain. Protection was attributed to the establishment of a cellular immune response dominated by antigen-specific T lymphocytes that produced interferon-γ and were cytotoxic towards infected cells. In addition, in heavily infected mice, hsp65-encoding DNA vaccination can switch the immune response from one that is relatively inefficient and produces bacterial stasis to one that kills bacteria. Thus, the DNA vaccine initially designed to prevent infection by *M. tuberculosis*, can also have a pronounced therapeutic action in mice [1]. Although the use of naked DNA is able to elicit an effective immune response in this experimental model of tuberculosis, the amount of plasmid required is very high. One of the reasons for the high doses of plasmid is that the extracellular delivery results in degradation by nucleases after conventional administration. Based on these observations, the use of delivery systems capable of protecting plasmid DNA represents an interesting strategy for vaccine administration avoiding vector loss.

We are exploring the use of PLGA microspheres for this proposal. In a preliminary work, we determined the safe doses of PLGA microspheres in rats and mice in order to optimize the maximal dose of this polymer. High doses of unloaded PLGA microspheres were related with TH2 response with the development of a fiber vascular capsule surrounding the microspheres. The reaction was not observed when lower doses were administered. Using an air-pouch model in mice, it was possible to quantify cell migration to the site of administration. Unloaded and antigen-loaded microspheres stimulated the macrophage migration.

In order to evaluate the use of this system as a vaccine carrier against tuberculosis, microspheres containing the recombinant protein hsp65, and also the gene encoding the hsp65 protein from *M. leprae* (pCDNA4-hsp65) were prepared by the W/O/W method. The recombinant protein and the plasmid were added to the inner aqueous phase. The polymer used was PLGA 50:50 (Resomer RG 505, MW 78,000, from Boehringer Ingelheim, Ingelheim, Germany). The vector pCDNA4 alone was also encapsulated and used as control group in the immunological studies. Macrophage-like cell lines J774 and J774-hsp65 (J774 cells transfected with a retroviral vector [pZIPNeoSV(x)] carrying the *M. leprae* hsp65 gene) were used for in vitro studies. The immunocytochemistry analyses performed 10 and 20 days after starting the treatment with microspheres showed that the encapsulation procedure did not affect the plasmid functionality. The immunization procedures were carried out using young adult BALB/c mice, 6 to 8-weeks old. Specific antibody levels were evaluated in serum and cytokine production was evaluated in supernatant of splenic cells culture or in lung cells homogenate using ELISA technique.

Our previous works with a DNA vaccine expressing hsp65 showed that, after intramuscular administration of 100 μg on 3 or 4 occasions at 3-4 week intervals, the immune response profile was predominantly of TH1 type (abundant IFN-γ, minimal IL-4). Concerning the encapsulation into PLGA microspheres, the first studies revealed that the better profile of cytokines and cell proliferation for selective TH1 response were obtained after administration of hsp65, plasmid-encoding-hsp65-loaded microspheres or a mixture of free plasmid and plasmid-loaded microspheres after three doses by intramuscular route. The control, unloaded microspheres and the vector-loaded microspheres are not able to induce any significant response. The plasmid encapsulation reduces the amount and number of administrations of DNA required to stimulate the desirable immune response.

In a second experiment, the recombinant hsp65 was incorporated into PLGA microspheres and administered by intratracheal route. Since most particles presented diameters smaller than 10μm, this contributed to improve their targeting to Antigen Presenting Cells (APC). BALB/c mice immunized with hsp65 in PBS or entrapped in microspheres presented serum production of antibodies specific to hsp65. Levels of IgG2a were similar between the groups that received the hsp65, 30 days after immunization. The protein encapsulation showed to be more selective to a TH1 response since the levels of IgG1, the isotype associated with a non-protective TH2 response, are higher in mice that received free hsp65 if compared to hsp65-loaded microspheres immunized mice. Spleen cells of mice immunized with hsp65-loaded microspheres, but not with free hsp65, were able to produce significant higher amounts of IFN-γ, if compared to non-stimulated control. In lung cell homogenate, high levels of IL-12 were observed in mice immunized with free hsp65 or entrapped into microspheres. These data suggest that hsp65-loaded microspheres are able to induce both systemic and local immune response after administration on lung mucous membrane.

In a third step, we have associated immunomodulator to the formulation of plasmid-loaded microspheres in order to improve the protective response. In the same formulation containing the plasmid, we added trehalose dimycolate (TDM) in the organic phase in order to obtain this substance dispersed in the polymer matrix after solvent evaporation. TDM is a glycolipid from the external layer of the cell wall of mycobacteria. It is a potent immunomodulator which, after microspheres entrapment, induces high levels of IL-6, TNF-α, IL-12, IL-10, IFN-γ and IL-4 production by lung cells after intratracheal administration and high production of nitric oxide (NO) in culture supernatants of bronchoalveolar lavage cells. In this latest experiment we demonstrated that BALB/c mice immunized with microspheres containing both pCDNA4-hsp65 and TDM presented serum production of antibodies specific to hsp65. Levels of IgG2a were higher than the controls while levels of IgG1 were not significantly different from vector plus TDM(alone)-loaded immunized mice. Production of IFN-γ, IL-12 and IL-10 in homogenate of lung cells from challenged mice was higher in mice immunized with microspheres containing both pCDNA4-hsp65 and TDM than the other groups.

We can conclude from our studies that PLGA microspheres are useful to protect the DNA and to improve the cytokine and antibody profile involved in the protection of tuberculosis. This new vaccine might be useful for both prophylactic and therapeutic proposals for human and veterinary therapy.

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PL-11 USE OF FOOD PROTEIN AS NEW EXCIPIENTS FOR BIOADHESIVE AND SUSTAINED RELEASE DRUG DOSAGE FORMS

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Introduction

The therapeutic activity of an active ingredient after its administration is the combined result of numerous processes linked both to the active ingredient itself along with the patient's physiological variables, and to the biopharmaceutical characteristics of the dosage form in which the drug is administered. Drug substances are seldom administered alone, but rather as drug dosage forms with one or more non medicinal agents that serve varied and specialized pharmaceutical functions. In a classical drug dosage form, the pharmaceutical excipients serve to increase the solubility, suspend, thicken, dilute, emulsify, stabilize, preserve and color the different drugs into a safe and convenient delivery system. However, numerous excipients now offer a regular controlled release of active ingredients for the purpose of optimizing administration. Thanks to these excipients, sustained, time or controlled release systems are developed to supply the optimal concentration of a drug for a longer duration and with more efficiency. In most cases, the excipients used are polymers with special properties, in particular gelling and swelling, which allow controlled release to be achieved.

A range of natural and synthetic polymers have been employed to control the release of drugs and other active agents. Such materials derive from various sources, the food industry being an important one. Among the different natural polymers, food proteins are very good candidates as functional excipients in the formulation of drug dosage forms.

The aim of this paper is to review the use of food proteins as excipients in the formulation of drug dosage forms and to highlight the *in vitro* and *in vivo* functional properties developed by the dosage forms formulated.

Origin of food proteins

Proteins represent a group of natural polymers. The basic structure of protein is a chain of amino acids that contain carbon, hydrogen, oxygen and nitrogen. All meat and other animal products are sources of proteins. These include beef, eggs, milk and milk products, but plant proteins, cheap and abundant are also of high interest.

Among the different sources of proteins used in food technology, dairy ingredients (caseins and whey proteins) and plant proteins (wheat and soy proteins) are the most interesting compounds because of their specific characteristics.

Apart from their nutritional properties, protein powders such as protein flours, concentrates and isolates have functional properties that contribute to their major roles of color, texture and flavor in food systems.

Functional properties of food protein

The functional properties of food proteins are fundamentally related to their physicochemical and structural properties such as size, shape, amino acid composition sequence, net charge, charge distribution, hydrophobicity/hydrophilicity ratio. According to the origin of the protein, the proportion and nature of the protein fractions and the proportion and nature of associated substances, protein flours, concentrates or isolates display various properties directly linked to the functional properties of the proteins they contain.

These functional properties include the following :

(i) Sensory and kinesthetic properties (e.g. flavor, odor, color, texture),

(ii) Hydration properties (protein-water interaction) : these include dispersibility, swelling, adhesion, solubility and viscosity,

(iii) Rheological properties : these are involved in processes such as precipitation, gelling and the formation of structures,

(iv) Surface active properties: e.g. surfactant, emulsifying, foaming, fat-binding properties,

(v) Other properties such as adhesive, cohesive, film and fiber making.

Pharmaceutical formulation can profit from the functional properties of food proteins by developing new controlled release drug dosage forms.

Formulation of matrix tablets

Matrix (monolithic) systems are possibly the easiest devices for controlling the release of drugs.

Generally the active agent is present as a dispersion within the polymer and the matrix systems are typically formed by the compression of a polymer/drug mixture. Food proteins have been used to some extent as carriers in sustained release matrix tablets since they are readily compressed and have gelling and water absorbing properties.

Examples of matrix formulations have been published using casein and modified casein, milk protein, soy bean protein, egg albumin and serum albumin. The mechanism of drug release was found to be a combination of diffusion, swelling and erosion. The *in vitro* drug release rate was controlled and directly related to the protein nature, amount and conformation, rate was unaffected by changes in pH and presence of enzyme.

The pharmacokinetic analysis following administration of matrix protein tablets showed sustained release absorption.

Formulation of bioadhesive drug dosage form

A drug delivery system with bioadhesive properties is in intimate contact with the absorptive surface, therefore reducing the problems of dissolution and drug absorption by increasing the concentration gradient across the mucosa. Milk protein concentrates allow the formulation of buccal tablets that will remain in contact with the oral mucosa while providing controlled release characteristics over a prolonged period of time.

Bioadhesive buccal tablets with different drugs have been developed and tested *in vitro* and *in vivo*. The in vitro dissolution kinetics showed a sustained release of the drug. The bioadhesive form placed on the buccal mucosa showed a delay while an equilibrium between release from the dosage form and absorption by the mucosa was set up. The bioadhesive buccal tablet exhibited a sustained release profile, the concentration increasing progressively until the end of bioadhesion determined either by disintegration time or by detachment time if the tablet fell off.

Specific bioadhesive tablets for other routes of administration such as the vaginal route have also been studied.

Encapsulation

A typical approach to controlled release is to encapsulate or contain the drug entirely within a polymer film or coat. Proteins have been shown to present a significant potential as wall materials for controlled and sustained release microcapsules with different drugs.

Several methods have been reported for the preparation of micro and nanoparticules from raw materials. Most methods involve the application of emulsion technologies or coacervation or controlled desolvation. For all the techniques, a subsequent treatment either by heating or by chemical cross-linking agent is necessary to stabilize the particles.

Most of the research has been focused on different albumins. Suitability of milk proteins and bovine caseins as wall materials has been investigated, and the results indicated that the microparticules formulated exhibited properties desired for controlled core release. The feasibility of preparing legumin and gliadin nanoparticles have been studied. Stability of the preparation, degradation kinetics and in vitro release profiles with different drugs have been investigated. Recently, the concept of using whey proteins as microencapsulation agents has been established. Whey proteins have good encapsulation properties for volatile and nonvolatile core materials.

Formulation of films

In food technology, an edible coating or film has been defined as a thin, continuous layer of edible material formed or placed on or between food or food components. Film formation properties can be applied to the pharmaceutical formulation.

Food proteins, especially soy and whey proteins, possess several functional properties important for biofilm formation. Different pharmaceutical applications of biofilm as coating materials have been investigated such as aqueous pseudolatex of zein for film coating of solid dosage forms or gliadin films as a carrier for controlled drug delivery.

In all cases, the release rate of drug was retarded and was controlled by the protein biofilm amount and composition.

Conclusion

The different studies performed have proven the feasibility of using food proteins from different origins (whole milk, soy bean, wheat…) as excipient for drug dosage forms. They strongly influence the properties of the dosage forms obtained. They present a high potential as sustained release, controlled release, bioadhesive, wall material or film coating excipient. Because of their specific functional properties, naturally occurring proteins form a novel class of easy to use and to obtain, safe and inexpensive excipients.

PL-12 CONTROL OF FORMULATION AND PROCESSING VARIABLES TO ACHIEVE DESIRED PROPERTIES OF MATRIX MICROSPHERES

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Introduction

Matrix microspheres are rugged and versatile entities to achieve control of drug release in a predictable manner. To some degree, the site, the onset, the intensity and the duration of release can be controlled for oral, topical and other dosage routes. As with any manufacture of any product, there are identifiable variables in the process and in the formulation of matrix microspheres. In order to obtain microspheres with characteristics that are suitable for a specific therapeutic objective, certain variables in the formulation and /or in the manufacturing process can be optimized to achieve the desired characteristics. If all the variables are appropriately identified and can be controlled, then microspheres can be reproducibly manufactured to meet certain specifications. It may not be possible to anticipate or control all variables in a particular formulation or process, but in our research we try to discover changes that modify the final product and devise ways to control at least the most important variables [1, 2, 3, 4]. It is the purpose of this lecture to present research findings that point to ways to control formulations and processes to achieve matrix microspheres that have the desired characteristics.

Formulation Factors

Identifiable factors in formulation of microspheres include the type and molecular weight of the polymer matrix, the solubility of the drug core in the dissolution environment, the solubility of the drug in the matrix, the size of the drug particles in the matrix, the concentration/proportion of drug in the polymer matrix, the homogeneity of drug particles in the matrix and in some cases the shape of the drug particles in the matrix [5].

Processing Factors

Identifiable factors in the emulsion-solvent extraction/evaporation (ESE) manufacturing process include the phase ratio, the physical and chemical characteristics of the phases, the mixing intensity , the temperature of processing , the rate of solvent escape from pre-microsphere globules and from the manufacturing vessel, and the emulsifiers and their concentration.

Formulation Control

Formulation control begins with selection of the form of the drug and its compatibility with solvents and polymers. Solubility in each phase is a prime consideration. If the drug is insoluble in the polymer solution phase and polymer, then particle size becomes very important. Examples will be shown of the effect of particle size on dissolution characteristics of the resulting microspheres [6]. Polymer characteristics affect microsphere dissolution. Examples of modifying the polymer matrix by the addition of another polymer of different character will be shown [7, 8].

Processing Control

Processing variables are more difficult to specify and control because the conditions during the formation of the microspheres are ill-defined. Temperature of precessing is readily defined and can be controlled by the use of jacketed, thermostated vessels. Although it is a major factor in controlling microsphere size, mixing intensity is much more difficult to specify and control since it depends on the shape and size of the processing chamber, the shape and size of the moving elements and their rates, and also on formulation factors. While most authors recognize the complexity of this problem, reports frequently state only "stirring speed " or stirring speed and propeller size. This is helpful for comparison in a fixed system, but is less useful for lab to lab evaluation. An important factor in any emulsified system is the phase ratio; it is no less so in the production of microspheres by ESE. Fortunately, the initial phase ratio is easy to define and specify. The ratio of the polymer phase to the continuous phase can affect the size distribution and other characteristics of the resulting microspheres. The viscosity of each phase affects the shearing forces that cause the formation of the initial polymer solution globules, therefore should be specified to control product characteristics. Also, polymer phase viscosity has been observed by our laboratory to affect dissolution characteristics of microspheres [9]. Research examples will be presented to show the importance of several of these controlling factors.

Conclusion

If formulation and processing variables can be identified and defined, they can point to methods of control that will allow tailoring the formulation and process to achieve desired product characteristics and will improve reproducibility.

Acknowledgment

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DD-01 CHARACTERIZATION OF A β- CYCLODEXTRIN-OMEPRAZOLE INCLUSION COMPLEX

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Introduction

Omeprazole (OME) is a well known anti-ulcerative drug that acts by inhibiting the acid gastric secretion by blocking the H+/K+ ATPase pump. The main pharmaceutical drawbacks are substantially related to the physicochemical instability to heat, light and acidic media, even with coated formulations. Moreover, the low aqueous solubility of OME is responsible for low dissolution rates and hence low bioavailability.

The cyclodextrins (CDs) are cyclic oligosaccharides composed of D-(+)-glucopyranose units which are connected with each other by the α-(1,4)-glucoside bond. It is known that CDs includes aromatic compounds, alkyl halides, gases, etc. as guest molecules in the CD cavity, resulting in the formation of inclusion complexes.

The complexation with cyclodextrins is an established procedure to improve the biopharmaceutical properties of drugs with poor water solubility.

Nuclear magnetic resonance (NMR) techniques are already known as powerful tools for the characterization of inclusion complexes. Information provided by these methods, includes:

A) confirmation of complex formation,
B) calculation of the stoichiometry of complex, and
C) establishment of the geometry of the new chlatrate.

The inclusion complex of OME and β-CD has been studied by proton NMR.

Experimental Section

- **Materials -** Omeprazole was obtained from Quimica Sintetica S.A. (Spain) and β-CD from Sigma.
- **Apparatus –** The H-NMR experiments were perfomed at 300 MHz using a BRUKER AVANCE DRX 300 spectrometer.
-

The testing temperature was regulated to 300 K. No internal H-NMR reference was added since the possibility of reference binding to the β-CD could not be excluded. A water line (4.75 ppm) was used as the reference.

Determination of the stoichiometry

Stoichiometry of OME-β-CD complex, by the continuous variation method, was based on H-NMR spectra obtained for OME and β-CD mixtures in which the total concentrations of the two species were maintained constant and the ratio (r) of the initial concentrations varied between 0 and 1. Under these conditions, if a physical parameter directly relates to the concentration of complex, it can be plotted as a function of r. Then, its maximal value corresponds to r = CD/(CD + OME), where CD and OME are the molar ratios of CD and drug in the complex.

- **Determination of the association constant by the method of Benesi-Hildebrand (linear)**

The association constant (k) can be evaluated from NMR parameters using graphical procedures in the case of 1:1 complex if one species is observed in the presence of a large excess of the other component**.**

Under these restricting conditions, the equilibrium constant can be rewritten as follows:

$$K \,.\, ([OME]_t - [C]) \,.\, [CD]_t = [C] \qquad (1)$$

When OME specie are observed in the presence of CD in large excess, and [C] (complex) is related to the chemical shift differences from a given signal from OME molecule by the following relation:

$$[C] = \Delta\delta_{obs} \,.\, [OME]_t \,.\, \Delta\delta_c^{-1} \qquad (2)$$

where $\Delta\delta_c$ represents the chemical shift defference (for a given proton) between the free molecule and the pure complex.

After some algebraic manipulation and elimination of $[OME]_t$ the basic equation becomes:

$$\Delta\delta_{obs} = k \,.\, [CD]_t \,.\, \Delta\delta.\ (1 + k \,.\, [CD]_t)^{-1} \qquad (3)$$

with the Benesi-Hildebran method, eqn. (3) was rewritten as follows:

$$(\Delta\delta_{obs})^{-1} = (k \,.\, \Delta\delta_c)^{-1} \,.\, ([CD]_t)^{-1} + (\Delta\delta_c)^{-1} \qquad (4)$$

Plots of $(\Delta\delta_{obs})^{-1}$ against $([CD]_t)^{-1}$ are linear. The slope, abcissa and ordinate intercepts are $(k \,.\, \Delta\delta_c)^{-1}$, $-k$ and $(\Delta\delta_c)^{-1}$ respectively.

- **Determination of k by non-linear method**

Defining the average number of cyclodextrin molecules bound per guest molecules as ñ gives the expression:

$$\tilde{n} = \Sigma\,(\text{CD bound to OME}) / \Sigma\,(\text{all OME}) \quad (5)$$

further definition of the quantity Σ (CD bound to OME) as CDb, The total bound cyclodextrin concentration, can be written:

$$\text{CDb} = \text{OME:CD} \quad (6)$$

The free cyclodextrin concentration, CD, is obtained from:

$$\text{CD} = \text{CD}_t - \tilde{n}\text{OME}_t \quad (7)$$

Using the term n and substituting the quantity CD in the following equation:

$$\Delta\delta_{bs} = [k \,.\, \text{CD}/(1 + k \,.\, \text{CD})]\,\Delta\delta_c \quad (8)$$

gives:

$$\Delta\delta_{obs} = \frac{k \,.\, (\text{CD}_t - \tilde{n}\text{OME}_t) \,.\, \Delta\delta_c}{1 + k \,.\, (\text{CD}_t - \tilde{n}\text{OME}_t)} \quad (9)$$

To solve equation 9, ñ is needed; this is derived from the continuous variation method.

Results and Discussion

- **H-NMR spectra Peak assignments**

Peak assignments of β-cyclodextrin were realized on the basis of previous works in the literature [29, 30].

Peaks assignments to the omeprazole were realized by one dimensional H-NMR for the aromatic signals and by two dimensional H-NMR (COSY and NOESY) for metil and metoxil groups.

- **Complex-Stoichiometry**

To elucidate the stoichiometry of the complex, the continuos variation method (JOB plot) was used. The plot of cyclodextrin signal of different molar fraction values "r" is reported in (figure 1) for the cyclodextrin in the complex.

The chemical shift (δ) is in direct relationship with the complex concentration expressed as functions of "r" (r= [βCD] / [βCD] + [OME]).

The JOB plot (figure 2) showed the maximum at r = 0.5, indicating a 1:1 stoichiometry

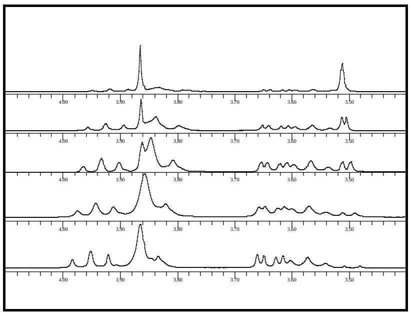

Figure 1

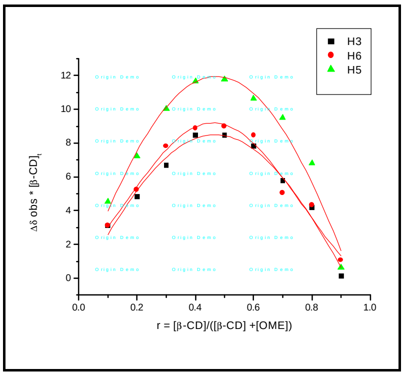

Figure 2.

- **Determination of the apparent equilibrium constant**

The equilibrium constant (k) was determined by the Benesi-Hildebrand method. The omeprazole concentration was maintained at 0.2 mM and the β-cyclodextrin concentration between 2 and 12 mM.

Some of the signals of the OME are divided because of the optical activity of the molecule [31-34], and so it can be possible to calculate the constant for the two isomers. For the low field isomer we obtained a k = 76.88 M^{-1} and for the up field isomer a k = 59.24 $M^{-1.}$ As this method it was not so precise, we determined the k values for the two isomers by a non-linear model described in material and methods. The signal of the proton utilized was that of the methyl at 569.5 Hz. For the low field signal, it was obtained a k value =94.14 M^{-1} and for the up field signal a k value = 57.45 M^{-1}

Conclusions

1- The stoichiometry of the complex cyclodextrin: omeprazole is 1:1 in deuterated solution (D_2O)

2- The association constants for the two isomers were 76.8 M^{-1} for the upfield one and 59.2 M^{-1} for the lowfield isomer, calculated by the Benesi-Hildebrand method.

3- For the non linear method the values of the association constants obtained for the upfield isomer were =94.1 M^{-1} and 57.4 M^{-1} for the low field one.

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DD-02 INCLUSION COMPLEXATION OF AN ESTER-TYPE LOCAL ANESTHETIC WITH β-CYCLODEXTRIN

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Introduction

Local anesthetics (LA) are widely used for regional anesthesia on surgery as well as for the local control of acute or chronic pain. Beyond the blockage of the voltage-gated sodium channels of axons, LA have non-specific action on other membrane ion-channels and some of their metabolites are toxic, specially those from the ester- type LA. In order to avoid these toxic side-effects and to improve the therapeutic index of the LA, drug-delivery systems have been developed.

Complexation with cyclodextrins (CD) may provide a good way to increase the solubility and the bioavailability of drugs. The cone shaped cavity of CD could easily accommodate hydrophobic molecules, such as the LA.

The objective of this work was to prepare and to characterize a Benzocaine (an ester-type LA)-β-CD complex. Solubility phase diagrams as well as thermal analysis and fluorescence spectroscopy indicated the most probably occurring stoichiometry of complexation.

Experimental methods

- **Materials**

Benzocaine (BZC) and β-Cyclodextrin (β-CD) were purchased from Sigma Chem. Co.

- **Sample preparation**

BZC/β-CD inclusion complexes were prepared by shaking equal amounts of the anesthetic and β-CD in aqueous solution. After equilibrium, the solution was freeze-dried and stored at – 20°C.

- **Phase-solubility studies**

The method is based in the solubility variation of the guest molecule upon increase in the concentration of the host [1]. Excess amount of BZC was added to aqueous solutions of increasing β-CD concentrations (0–10 mM). After a shaking period, at room temperature, an aliquot was filtered and the amount of soluble BZC was evaluated from the absorbance at 284nm. Plots of BZC solubility as a function of the host (β-CD) concentration allowed the determination of the BZC/β-CD molar ratio of complexation.

- **Differential Scanning Calorimetry**

Samples of BZC (10 mg), β-CD, BZC/β-CD physical mixture and the solid complex BZC/β-CD were run in a Universal V2.3D TA Instruments calorimeter, at a 10°C/min heating rate, over a 30-200°C range.

- **Fluorescence studies**

We monitored the changes in the intrinsic fluorescence (I_0/I) of BZC upon variation of BZC and β-CD concentration. Applying the Job plot analysis [2] we determined the stoichiometry of the complex.

Results and discussion

- **Phase-solubility studies**

In the concentration range of β-CD studied only soluble complexes were formed and the linear solubility isotherm obtained (Figure 1) demonstrates that a 1:1 BZC/β-CD molar ratio is achieved in solution at complexation. Besides, the solubility of BZC was increased from 4.4 mM (in water) to 10 mM, in BZC/β-CD complex.

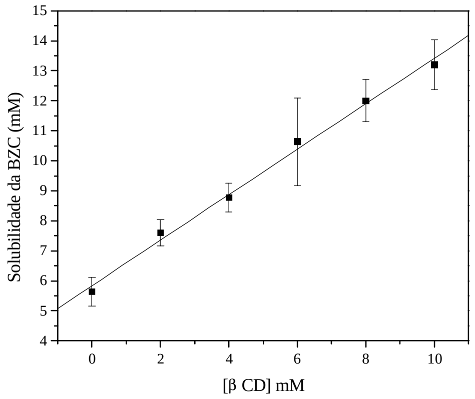

Figure 1 – Solubility isotherm of BZC under β-CD increasing concentration.

- **Differential Scanning Calorimetry**

DSC analysis showed that after complexation the inclusion compound looses its individual transitions, denoting the interaction at the molar ratio tested (Table 1). A broad transition was recorded for the complex in a temperature range between 60-120°C.

Table 1 – Transition temperatures obtained by Differential Scanning Calorimetry.

	Transition temperature (°C)
β-CD	115.9
BZC	87.9
BZC/β-CD -physical mixture	83.9 and 110.5
BZC/β-CD - complex	--

- **Fluorescence studies**

The fluorescence of BZC (I/I_0) increased in the presence of β-CD up to a 1:1 molar ratio (r = 0.5 in Figure 2), when the anisotropy of the molecule inside the host cavity is maximum and therefore the preferential molar ratio of the complex is reached.

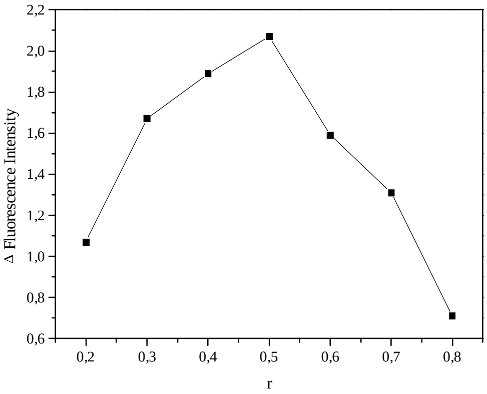

Figure 2 – Job plot: continuous variation method for the study of BZC/β-CD complexation, using the variation in the fluorescence intensity of BZC.

Conclusion

This study has shown that the ester-type local anesthetic Benzocaine could be complexed inside the β-cyclodextrin cavity. Altogether the results obtained are compatible with a preferential 1:1 BZC/β-CD stoichiometry of complexation.

Acknowledgements

This work was supported by "Fundação de Amparo a Pesquisa de São Paulo", FAPESP, Brasil.
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DD-03 DEVELOPMENT OF FORMULATIONS FOR PULMONARY ADMINISTRATION OF INSULIN – A PRELIMINARY STUDY

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Introduction

The control of insulin-dependent diabetes mellitus (IDDM) requires injections of insulin, which is an uncomfortable and expensive form of administration. The oral administration is the best and most easily accepted by the patients and also excludes an important side effect associated with insulin injection, the peripheral hyperinsulinism. However, due to the fast destruction by proteolytic enzymes, the hydrophilic nature of insulin and the complexation property when in solution, the diffusion through the gastrointestinal barrier is very low [4]. The insulin administration by pulmonary route has also been investigated. The high vascularization of the mucosa, enormous surface area (about 143 m^2 in adults), broad permeability characteristics in the alveoli due to a very thin epithelium and the reduced local inactivation of insulin allow to an efficient absorption. However, it has been previously reported that insulin permeability along the tracheobronchial tree varies as function of penetration distance, where rapid mucociliary clearance imposes an additional barrier [3].

In order to reduce some of these problems, the present study aims to develop a new formulation based on the microencapsulation of insulin complexed with cyclodextrins into PLGA microspheres.

Experimental methods

- **Materials**

Porcine Insulin (28 UI/mg), Poly(DL-lactic acid-co-glycolic acid) copolymer 50/50 (PLGA, Resomer RG 505), 2,6-Dimethyl-β-cyclodextrin (CD) were purchased from Sigma Chemical Co, Polyvinyl alcohol (PVA) were purchased from, Aldrich Chemical Co.

- **Preparation of complex insulin: dimethyl-β-cyclodextrin**

The complex drug–CD was carried out in an aqueous phase. Porcine Insulin and dimethyl-β-cyclodextrin were accurate weighted and solubilized in 125 μL PBS in the follow molar proportions 1:2, 1:3 and 1:5. The solutions were maintained under agitation for 4 h at room temperature.

- **Preparation of insulin/CD-loaded PLGA microspheres:**

PLGA microspheres were prepared by a double emulsion method. The aqueous phase containing insulin-CD in the concentration (10 mg%) of insulin) was added to 30mL of a PLGA solution in dichloromethane (7 mg/mL). The mixture was stirred at 13,500 rpm (Ultraturrax T25 –IKA) for one min. to form the first emulsion (W/O). This first emulsion was added to a volume of 100 mL of an aqueous solution of PVA (5%W/V) under agitation at 900 rpm (Eurostar – IKA) at room temperature until evaporation of the organic solvent. The microspheres were collected by centrifugation (23,500 x g, Himac CR21 – Hitachi) for 10 min. The obtained microspheres were washed three times with water and freeze dried [2].

- **Determination of size distribution**

The particles were dispersed in water and the size distribution was determined by laser diffractometry using a Cilas equipment (model Analyser 1064C) [2].

- **Determination of insulin loading**

10 mg of microspheres were precisely weighed and dissolved in 1 mL of acetonitrile for polymer solubilization in an Eppendorf tube. The polymer solution containing suspended insulin:dimethyl-β-cyclodextrin was centrifuged at 10,000 x g for 5 min. The remaining pellet was dissolved in 1 mL of HCl 0.1 N. The concentration of insulin solution was determined by HPLC method using an UV detector (HPLC Waters) [1,2].

- **Pulmonary administration of insulin**

Female mice, 28-30 g were anaesthetized by intraperitoneal injection of 2,2,2, tribromoethanol (300mg/Kg). After tracheal incision the formulations were administered into the lungs (approximately 0.1 mL of solutions) by means of a syringe.

- **Measurement of plasma glucose**

The plasma glucose concentration was obtained after centrifuging the amount of fresh blood sample in an Eppendorf tube with fluoride for 1 min at 10,000 rpm. The glicemia was measured by an enzymatic method (*in vitro* diagnostic – enzymatic glucose – CAT:10360).

Results and discussion

The effect of the molar proportion of insulin:CD on size distribution of the microspheres of PLGA was investigated. There was no significant difference for the preparations. The Table I shows the average diameter of the microspheres.

Table I. Average diameter of insulin-loaded PLGA microspheres (n=2).

Microspheres prepared with insulin:CD	Average diameter (μm)
1:2	2.14
1:3	2.35
1:5	2.53

Table II. Insulin content in PLGA microspheres (n=2).

Microspheres prepared Insulin:CD	Insulin loading (%)
1:2	2.99
1:3	11.75
1:5	14.76

The Table II shows that it was observed an increase in insulin encapsulation when the molar proportion of CD increased. The molar proportion 1:5 insulin:CD showed to be the optimal proportion and it was selected for *in vivo* test.

The hypoglycemic response following intratracheal was achieved after administration of 3 UI kg^{-1} insulin in a solution preparation (Ins), insulin-loaded microspheres (Ins-M), and Insulin/CD-loaded microspheres (Ins/CD-M). Phosphate buffered saline (PBS) and unloaded microspheres (Unl-M) were used as control. The Figure 1 shows the hypoglycemic profile.

After administration of PBS or unloaded microspheres it was not observed a significant reduction of initial glycemia. Free insulin and Insulin-loaded microspheres, complexed or not with cyclodextrin, showed a significant decrease in the glucose plasma level even though three hours after administration.

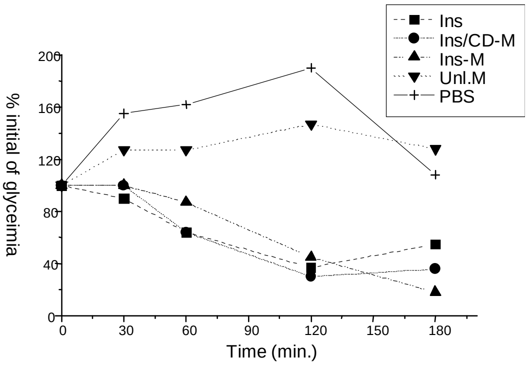

Figure 1– % of plasma glucose after intracheal administration of free insulin and insulin-loaded microspheres. (mean ± 14.5 SD, n=3).

Conclusion

This preliminary work revealed that insulin-loaded microspheres have a potential as a vehicle for pulmonary administration. The complexation with cyclodextrins have some other advantages related to the stability improvement drug release control which were not presented in this study.

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DD-04 PHYSICO-CHEMICAL STUDY ON THE INCLUSION COMPLEXATION OF 17-β-ESTRADIOL WITH HYDROXYPROPYL-β-CYCLODEXTRIN

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Introduction

Cyclodextrins (CDs) are a group of cyclic oligosaccharides consisting of 6, 7 or 8 glucopyranose units, usually referred as α-, β-, γ-cyclodextrins, respectively. This molecules have a hydrophilic exterior and a hydrophobic internal cavity. In aqueous solutions, CDs are capable of forming inclusion complexes with many drugs by taking up a whole drug molecule into the cavity. Inclusion complexation leads to changes in the physicochemical properties of the guest molecules, which can improve the molecular stability and bioavailability of several drugs [1,2].

Many metabolic important compounds such as hormones have very low solubility in aqueous solutions. Various approaches have been taken to use these compounds in transdermal systems. Two most frequently used approaches are: (1) predissolving the compounds in organic solvents, which affect the barrier function of the skin and (2) using "carrier" molecules to facilitate the dissolution and to enhance cutaneous absorption of this compounds. Additionally, one class of carrier molecules is the cyclodextrins.

Therefore, the present study was designed to prepare and characterize complex of 17-β-estradiol with hydroxypropyl-β-cyclodextrin.

Experimental methods

- **Materials**

Hydroxypropyl-β-cyclodextrin (HP-β-CD) was obtained from TRAPPSOL (Gainesville, FL, USA) and 17-β-estradiol was obtained from SIGMA (St. Louis, MO, USA).

- **Phase solubility studies**

Solubility studies were conducted according to the method of Higuchi & Connors [3]. Excess amounts of estradiol (4 mM) were added to aqueous solutions containing various concentrations of HP-β-CD, ranging from 4.0 mM to 50.0 mM and were shaken at 25ºC for 24 h. After agitation, aliquots of the suspension were filtered through a 0.45-μm membrane filter (Milipore), diluted and analyzed at 280 nm using a spectrophotometer (HITACHI U-3501).

- **Determination of the stoichiometry between estradiol HP-β-CD**

A continuous variation method was used to determine the stoichiometry of complex. Pre-made stock solutions (55.0 mM) of estradiol and HP-β-CD in methanol:water (10:90) were mixed in different molar ratios before measurements and were observed using spectrophotometer at 280 nm.

- **Differential scanning calorimetry measurements**

DSC measurements were conducted on estradiol, HP-β-CD, 1:3 complex and respectively equimolar physical mixture. Samples were heated in open aluminum pans from ambient temperature to 250ºC at heating rate of 5ºC/min and using argonium as purging gas.

- **Nuclear magnetic resonance measurements**

NMR was carried out using a BRUKER DPX spectrometer operating at 300 MHz. Samples were diluted in D_20/deutered methanol (50:50). Tetramethylsilane was used as internal reference.

Results and discussion

The phase solubility diagram of estradiol in different concentrations of HP-β-CD is shown in Figure 1. A linear increase of the estradiol solubility was observed as a function of HP-β-CD concentration. This curve can be classified as A_L type and it is possible to hypothesize a complex 1:1 stoichiometry. However, a continuous variation method was used to determine the stoichiometry of the complex, which indicates a 1:2 stoichiometry relationship, as shown in Figure 2. The high apolarity of estradiol molecule lead to consider this stoichiometry ratio, i.e., the complex is formed by 1 molecule of the drug entrapped by 2 molecules of HP-β-CD.

The calorimetric curves obtained for estradiol-HP-β-CD system are shown in Figure 3. The thermal curve of estradiol (T_{peak} = 182.6ºC) indicates its crystalline anhydrous state. A broad endotherm peak was observed in the HP-β-CD thermogram, which is probably associated with water loss.

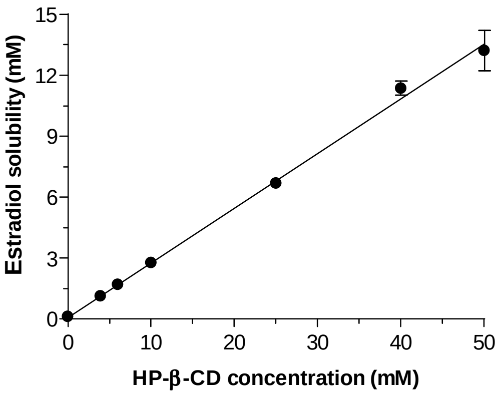

Figure 1– Solubility diagram of estradiol in the presence of HP-β-CD. Values, in duplicate, are showed as means ± S.D. r=0.9988.

A new transition at about 158.9ºC can be observed in the physical mixture. This transition could be due a possible complex formation during the physical mixture procedure. Marked broadening and down-shift were observed in the complex thermal curve (T_{peak}=140.3ºC), indicating inclusion complex formation through weak interactions between the estradiol and HP-β-CD.

Table 1. Solubility and stoichiometry of estradiol/HP-β-CD complex.

Increase of solubility	K_C (1:1)	Stoichiometry ratio (E/CD)	Molar ratio (E/CD)
104 fold	4.16 M^{-1}	1:2	1:3

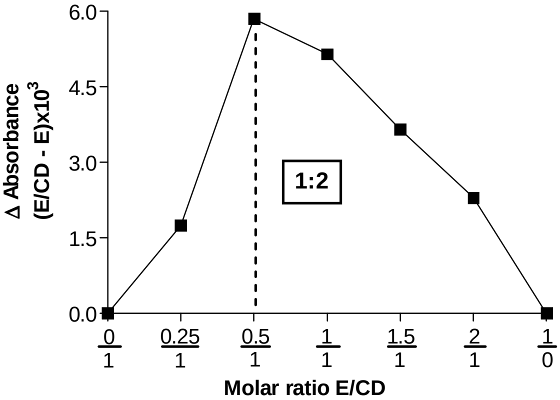

Figure 2– Continuous variation diagram for estradiol (E):estradiol/HP-β-CD (E/CD).

^{1}H-NMR spectroscopy was able to assure the existence of the complex in solution. The paramagnetic shift observed for H-3 proton of HP-β-CD, localized at the internal surface of CD cavity, indicates that estradiol molecule created paramagnetic anisotropy effects in the interior of the cavity due to weak interactions (van der Waals forces), which is an evidence of inclusion complexation. Additionally, the diamagnetic shift observed for H-2 and H-4 proton of estradiol (aromatic ring) is probably related to the association with the oxygen atoms of the CD in the internal cavity, while the paramagnetic shift observed for H-18 characterizes a deshielding effect from van der Waals forces between drug and carbohydrate chains.

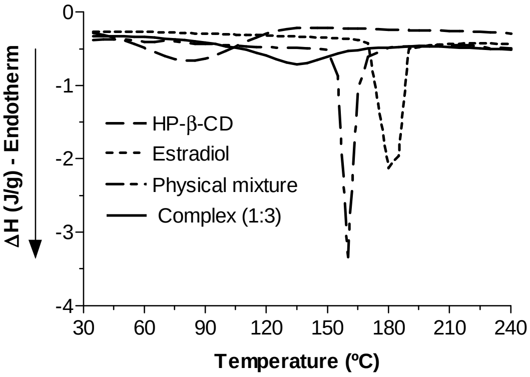

Figure 3– DSC thermograms of estradiol, HP-β-CD, 1:3 complex and respectively equimolar physical mixture.

Conclusion

The results obtained in this work proves the complexation of estradiol by HP-β-CD. However, a weak interaction was observed between the drug and CD. Additionally, a 1:2 complex formation of estradiol/HP-β-CD produces a marked increase of water solubility of the drug that lead to consider its potential use for transdermal delivery of estradiol.

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DD-05 PREPARATION IN SOLID STATE OF AN ASSOCIATION COMPLEX BETWEEN MEGLUMINE ANTIMONATE AND CYCLODEXTRIN

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Introduction

The pharmaceutical and food industry have been using saccharides and polysaccharides as additives for their ability to modify the properties of aqueous environment. Nowadays, the oligosaccharides, cyclodextrins, are one of the mostly used additives in the above industrial applications. Cyclodextrins have been mainly used to enhance the solubility of sparingly water-soluble drugs and also to improve their absorption, chemical stability and biodisponibility. Intermolecular interactions, such as hydrogen bonding, determine many physical properties of these pharmaceutical formulations. We have studied the interaction between β-cyclodextrin and meglumine antimonate using thermal analysis (TG, DTG and DSC) and X-ray patterns diffraction (XRD). Meglumine antimonate is one of the first line drugs for the treatment of leishmaniasis. However, its use is often limited by its high systemic toxicity and the lack of reproducibility of its formulation. Its association with cyclodextrin may helps to improve these aspects. In this work, we report that meglumine antimonate interacts with cyclodextrin as an association complex. More strikingly, we observed an interaction of antimonate meglumine with β-cyclodextrin in the solid state following their physical mixture.

Material and Methods

- **Preparation of meglumine antimoniate**

Meglumine antimonate (MA) was prepared as previously described [1].

- **Preparation of the association complexes**

The association complex of meglumine antimonate (AC) with β-ciclodextrin (β-CD) was prepared at molar ratio of 1:1 in aqueous medium, by co-precipitation method as related in the literature [2].

The physical mixture (PM) was prepared at the same molar ratio.

- **General experimental techniques**

TG and DTG curves were registered on a TGA-50 Shimadzu thermogravimetric analyzer and DSC curves on a DSC-50 Shimadzu differential scanning calorimeter. An heating rate of 10 ^{o}C/min under dynamical N_2 atmosphere was used. X-ray pattern diffraction, XRD, was obtained using a X-ray Rigaku spectrometer, model Geigerflex, fitted with a LiF monochromator and employing Cu K-alpha radiation. Antimony content was determined by atomic absorption using a Hitachi Z8200 spectrophotometer.

Results and Discussion

Characterization studies of association complex usually use the physical mixture as a control. This is why both association complex and physical mixture were studied in the present work. We report the evolution of DSC and XRD parameters as a function of time

- **Thermal analysis**

The figure 1 shows the DSC curves of physical mixture MA/β-CD at different times after the preparation. We observed that the system reachs equilibrium only on the fifth day after mixing.

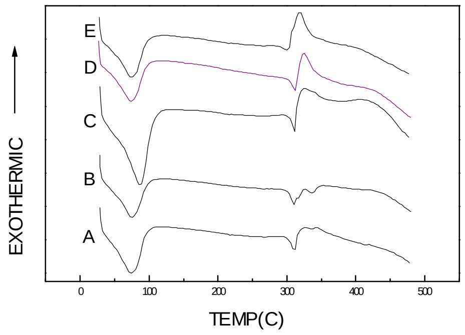

Figure 1. DSC curves of physical mixture as a function of time. (A) 0 hour, (B) 4 hours, (C) 1 day, (D) 5 days, (E) 8 days.

The TG and DTG curves for PM presented two decay steps. The first around 60°C is due to loss of water molecules (data not shown).

The second is due to the thermodecomposition of the PM. The TG curve showed a thermal stability between 165–250°C that was not observed with MA (data not shown). Therefore, our data shows that the thermal stability of MA is improved upon mixture with β-CD.

The DSC (figure 2) of the PM exhibited an endothermic peak around 70°C that corresponds to the loss of water molecules, an endothermic peak around 312°C due to the melting and a exothermic peak around 327°C due to a thermodecomposition of the PM.

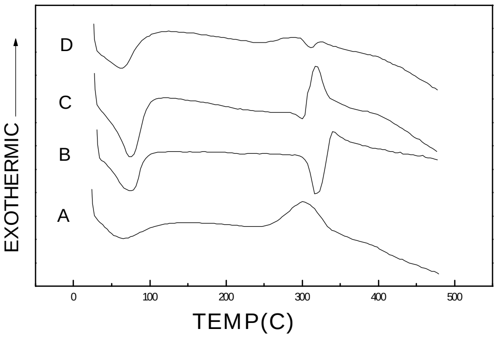

Figure 2. DSC curves of (A) antimonate meglumine, (B) β-cyclodextrin, (C) the physical mixture, (D) the association complex (by co-precipitation).

It is noteworthy that DSC curves did not show a superposition of the thermal events of the MA and β-CD. These experiments revealed that there is an interaction between meglumine antimonate and βciclodextrin in the solid state following their physical mixture. The DSC curve of AC, when compared to that of PM, show that AC and PM are different species.

- **X-ray analyses**

The figure 3 shows the XRD pattern diffraction of PM at different times following the preparation. We observed alteration of the crystalline arrangement during the first five days. Later, the XRD pattern remained stable. The X-ray diffraction parameters, displayed in Table 1, indicate that PM and AC are different species and are less crystalline than β-CD.

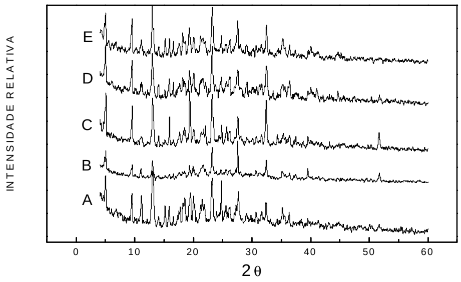

Figure 3. X-ray diffraction of physical mixture as a function of time. (A) 0 hour, (B) 4 hours, (C) 1 day, (D) 5 days, (E) 8 days.

Table1. X-ray diffraction parameters of βcyclodextrin (β-CD), physical mixture (PM) and association complex (AC) (by co-precipitation).

β- CD		PM		AC	
d(Å)	I/I_0	d(Å)	I/I_0	d(Å)	I/I_0
17,7	61	17,7	77	---	---
9,2	37	9,2	63	---	---
7,9	41	---	---	7,9	72
---	---	---	---	---	---
6,8	100	6,8	85	6,8	100
5,8	20	---	---	---	---
5,5	38	---	---	---	---
5,3	17	---	---	---	---
5,0	27	---	---	---	---
4,8	57	---	---	---	---
4,5	26	4,5	49	---	---
---	---	4,4	44	4,4	64
4,3	49	---	---	---	---
---	---	---	---	4,2	64
4,1	53	---	---	---	---
---	---	---	---	---	---
---	---	3,8	100	3,8	55
3,7	69	---	---	---	---
3,6	19	---	---	3,6	45
---	---	---	---	---	---
3,4	51	---	---		
3,3	34	---	---	---	---
3,2	32	3,2	66	---	---
3,1	21	---	---	---	---
3,0	21	---	---	---	---

Conclusion

The physical mixture led to the formation of an association complex between β-CD and meglumine antimonate by reaction in solid state. The resulting compound exhibited different thermal behavior and X-ray diffraction parameters when compared to the compound obtained by the classic method of co-precipitation. Our data suggests that reaction in solid state between drugs and cyclodextrins may be used in the preparation of pharmaceutical formulation.

Acknowledgments

This work was supported by CAPES, CNPq and FAPEMIG (Brazil).

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DD-06 MORPHOLOGICAL ANALYSIS OF THE INCLUSION COMPLEX BETWEEN 3-(4’-BROMO-[1,1’-BIPHENYL]-4-YL)-3-(4-BROMOPHENYL)-N,N-DIMETHYL-2-PROPEN-1-AMINE AND β-CYCLODEXTRIN

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Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides named α, β and γ-CD, which have a relatively hydrophobic central cavity and a hydrophilic outer surface. A number of guest molecules form stable host-guest inclusion complexes with cyclodextrins [1]. This may alter many physicochemical properties of the included molecule and the inclusion complexes can be exploited in the pharmaceutical field to enhance the stability, solubility and bioavailability of various drugs.
3-(4’-Bromo-[1,1’-biphenyl]-4-yl)-3-(4-bromophenyl)-N,N-dimethyl-2-propen-1-amine (BBAP) is a drug with promising pharmacological activities against *Trypanosoma cruzi*, *Leishmania amazonensis* and mycobacteria [2,3].

Goals

In this study our main goal was to analyze the morphology of the inclusion complex and of the physical mixture between BBAP and β-CD by Scanning Electron Microscopy (SEM).

Methodology

The inclusion complexes of BBAP/β-CD were prepared as follow. BBAP and β-CD in the molar proportion of 1:1 and 1:2 were dissolved in a mixture of water/ethanol (30:100) and the solvent was evaporated at 80°C under reduced pressure. Physical mixtures between BBAP and β-CD in the molar proportion of 1:1 and 1:2 were uniformly and gently mixed with care to avoid any grinding action in the mixture. Either BBAP, inclusion complexes or the physical mixture were totally dried under high vacuum at room temperature and stored in a desiccator over silica. The spectroscopy techniques of Differential thermal analysis (DTA), X-ray powder diffractometry, infrared spectroscopy, induced circular dichroism (ICD) and UV-Vis spectroscopy were previously used to characterize the preparations [4]. The morphology analysis of the BBAP, β-CD, physical mixtures (BBAP/β-CD 1:1 and 1:2) and of the inclusion complexes (BBAP/β-CD 1:1 and 1:2) were performed by means of Scanning Electron Microscopy (SEM) using a JEOL JSM – T300 Scanning microscope. The samples were mounted on aluminum stubs using double sided sticky tab and vacuum coated with gold for 100 seconds to render them electrically conductive.

Results

In the morphological analysis, typical crystals of β-CD were found in many different sizes (Figure 1). BBAP showed an amorphous structure by SEM (Figure 2) as well as by X-ray diffraction. There was an agglomeration of BBAP around the β-CD, both in the BBAP/β-CD 1:1 and 1:2 physical mixtures (Figure 3 and 4). In the case of the co-evaporated inclusion complex BBAP/β-CD 1:1 and 1:2, a compact structure formed by the aggregation of the crystals it was observed (Figure 5 and 6).

Conclusions

The combined spectroscopy techniques indicated the formation of a real inclusion complex between BBAP and β-CD by co-evaporation and no complex was obtained in physical mixture of the compounds. The scanning electron microscopy (SEM) revealed distinct morphological differences among the different preparations contributing to the characterization of the physical mixtures and of the inclusion complexes BBAP/β-CD 1:1 and 1:2.

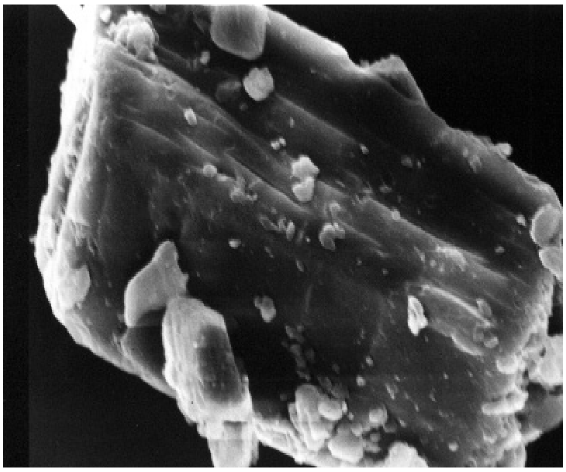

Figure 1. SEM of β-CD – Magnification of 1500X

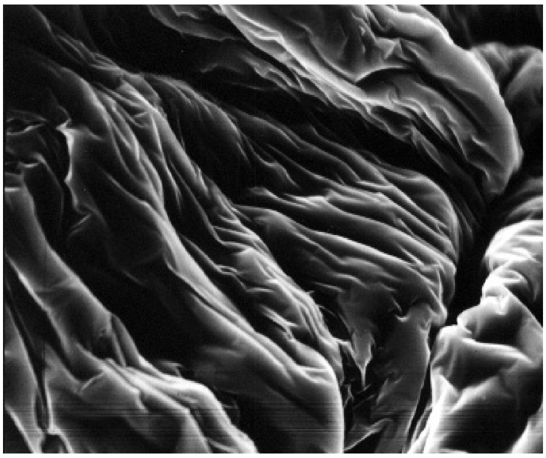

Figure 2. SEM of BBAP – Magnification of 1500X

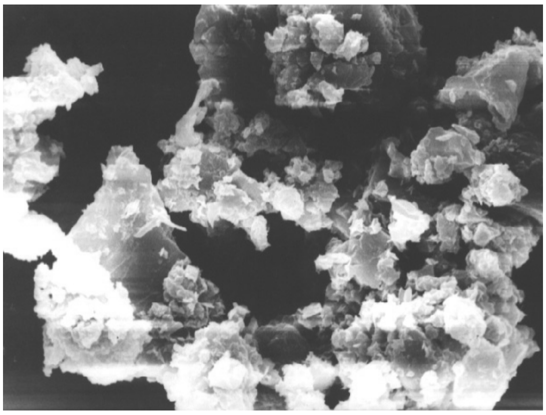

Figure 3. Physical mixture BBAP/β-CD 1:1- 1500X

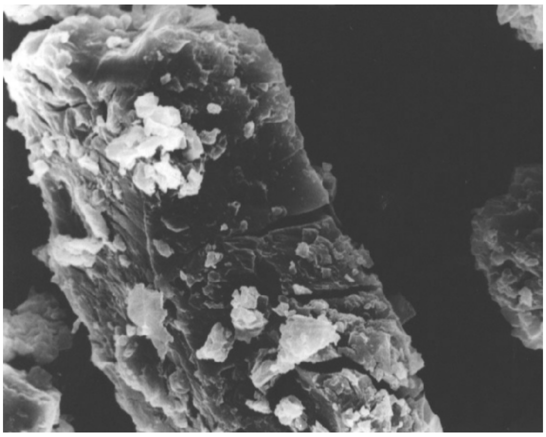

Figure 4. Physical mixture BBAP/β-CD 1:2- 1500X

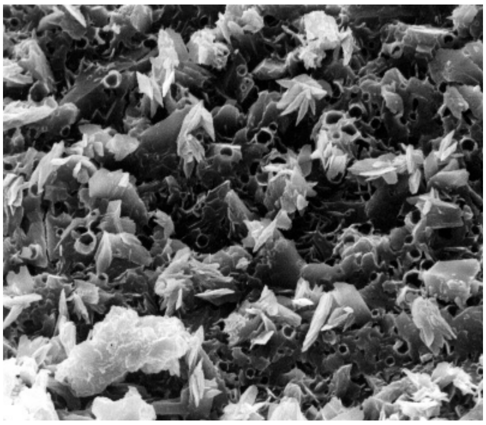

Figure 5. SEM of the Inclusion complex BBAP/β-CD 1:1 - Magnification of 1500X

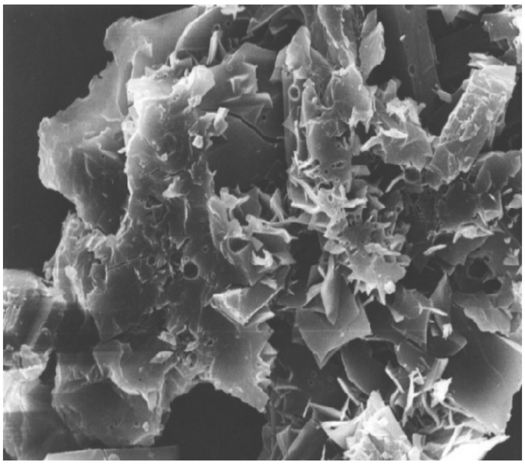

Figure 6. SEM of the Inclusion complex BBAP/β-CD 1:2. Magnification of 1500X

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DD-07 STABILITY TEST OF PERORAL VACCINE DELIVERY SYSTEM BASED ON NON-IONIC SURFACTANT VESICLES (NIOSOMES)

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Introduction

Presently most vaccines for protection against both superficial and systemic pathogens are administered parenterally in order to avoid antigen degradation by gastric acidity and proteolytic enzymes in the gut. However, the majority of infectious diseases in encountered through the large area of the body's mucosal surfaces, e.g. gastrointestinal tract, respiratory tract and uro-genital tract [1]. These surfaces are protected by their own secretory immune system which cannot be activated efficiently by the parenteral route, including ease and convenience of administration, increased patient compliance, reduced cost and minimal side effects and thus a greater possibility of frequent boosting.

Niosomes are vesicles composed of non-ionic surfactants consisting of one or more lipophilic bilayers, which encapsulate an aqueous medium [2]. They are able to encapsulate both hydrophilic and lipophilic substances and may be used for protecting proteins or peptides administered perorally [3]. For this route of uptake the stability of the drug delivery system within the gastric pH and bile salts is of crucial importance.

The aim of the investigation is to evaluate the potential of a new vaccine delivery system based on non-ionic surfactant vesicles, which is strongly related to its stability against compounds such as bile salts present in the intestinal fluids and the gastric pH. The sucrose ester surfactants used in this study form gel state bilayers at 37ºC and have been chosen due to their easy biodegradability and very low toxicity.

Materials and Methods

The vesicles were prepared from sucrose ester surfactants, cholesterol and dicetyl phosphate. The sucrose ester surfactant was kindly given by Mitsubishi-Kagaku Foods Corp., Tokyo, Japan. All other chemicals were of analytical grade.

- **Preparation of vesicles. Sonication method**

Stock solutions [150 mM] of the surfactants in a chloroform/methanol mixture were vigorously mixed. The organic solvent was removed with N_2 current for 6 hours. 5 ml of phosphate buffered saline (pH 7.4) were added to the surfactant film and the samples were heated in a water bath (80°C) for 15 minutes. The samples were sonicated for 60 s using a sonication bath at 80ºC *Fungisonic, FUNGILAB S.A.*, Barcelona, Spain. The procedure resulted in multilamellar vesicles, which were cooled down to 25°C directly after sonication.

Characterization

- **Vesicle size**

The diameter of the vesicles were obtained by Laser light scattering, employing a ***MALVERN Mastersizer X*** (MALVERN Instruments Ltd., Worcestershire, GB) with a He-Ne-laser (λ=0.63 nm).

- **Light polarization microscopy**

The samples were investigated by means of a Carl-Zeiss, standard polarization microscope (Carl-Zeiss Germany). A small droplet of the vesicle suspension was placed on a glass microscope slide and covered with a glass cover slip. The samples were examined for phase separation, vesicle formation and crystal formation. Large multilamellar vesicles were recognised by what is known as Maltese crosses.

- **Stability tests**

The stability of the vesicles at pH 2 and in a bile salt solution at a concentration above the critical micelle concentration [4] was monitored at 37 °C by laser light scattering. Vesicles formulations were incubated in deoxycholate, (SIGMA, St. Louis (MO), USA) dissolved in phosphate buffered saline.

Results

Light polarization microscopy study revealed that the suspension prepared from sugar ester surfactant was heterogeneous in terms of vesicle shape and size.

The average size of the vesicles varied between 10 μm to 15 μm.
The stability test with deoxycholate at a concentration above the critical micelle concentration showed no significant changes of the vesicle size over 5 hours. The same holds also for the pH 2 over 3 hours.

Conclusions

The particle size of the vesicles may lead to think that they are able to be uptake by the immune system overlying the intestinal mucosa.
Vesicle compositions seem to be stable at pH 2, the diameter of the vesicles did not change within three hours. The presence of bile salts did not induced aggregation or solubilization of the samples during five hours.
The peroral use of drug delivery systems based on non-ionic surfactant vesicles seems to be possible.

Acknowledgements

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DD-08 LONG-LASTING CARDIOVASCULAR EFFECTS OF LIPOSOME-ENTRAPPED ANGIOTENSIN-(1-7) AT THE ROSTRAL VENTROLATERAL MEDULLA

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Introduction

Several recent studies indicate that angiotensin-(1-7) (Ang-(1-7)), a peptide of the renin-angiotensin system (RAS), may act as an important neuromodulator at the the rostral ventrolateral medulla (RVLM), a brain medullary area related to the tonic and reflex control of arterial pressure [1]. Micro-injection of Ang-(1-7) in the RVLM was found to increase the arterial blood pressure and it is suspected that chronic increased levels of Ang-(1-7) at the RVLM may contribute to hypertension. Until now, it has not been possible to confirm this hypothesis, mainly because of the short half-life of the peptide which prevents the evaluation of its long-term effects. To address this question, injectable microreservoirs, that could act as sustained release systems in this specific site of the brain, have to be developed.

In this work, we evaluated the potential of liposomes for the sustained release of Ang-(1-7) at the RVLM. More specifically, liposome-encapsulated Ang-(1-7) was micro-injected unilaterally in the rat RVLM and the effects on blood pressure and heart rate were evaluated by telemetry.

Experimental Methods

- **Materials**

Ang-(1-7) was obtained from Bachem (Los Gatos, CA, USA). L-α-distearoylphosphatidylcholine (DSPC) and cholesterol (CHOL) were purchased from Sigma Co. (St. Louis, MO, USA). Distearoylphosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG2000) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). 1,1´-dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Molecular Probe (Eugene, OR, USA).

- **Animals**

Wistar rats (male weighing 250±10 g) were used throughout the experiments. Before and after surgery, animals were kept in a light- and temperature-controlled room. A 12:12-hour light-dark cycle was maintained with lights at 6 AM.

- **Preparation of liposomes**

The encapsulation of Ang-(1-7) in liposomes was performed according to the Dehydration Rehydration Vesicles (DRV) method [2] and followed by extrusion of the DRV suspension through polycarbonate filters of 200 nm pore size, using an Extruder (Lipex Biomembranes, Vancouver, BC, Canada)[3]. Peptide-containing liposomes were separated from unencapsulated peptide by dialysis and then sterilized by filtration. A lipid composition of DSPC, CHOL DSPE-PEG2000 at a molar ratio of 5:4:0.3 was used. The amount of encapsulated peptide was determined using the intrinsic fluorescence of Ang-(1-7). An encapsulation efficiency of 12% was achieved and a ratio of encapsulated peptide to lipid of 0.03 (w/w). Analysis of the size of the liposomes by quasi-elastic laser light scattering gave an average vesicle diameter of 0.19 μm.

Liposomes, labelled with the lipophilic fluorescent dye, DiI, were prepared essentially according to Claassen [4].

- **Micro-injection of liposome preparations**

Liposome-encapsulated Ang-(1-7) (LAng) was unilaterally micro-injected (35 ng/200 nL) into the rat RVLM with an injection needle (30 G) that was slowly inserted in the brain tissue through the dorsal surface, using the same extereotaxis coordinates (1.8 mm anterior, 1.8 lateral to the obex, and just above the pia-mater). Empty liposomes (Lemp) or diI-labelled liposomes were micro-injected similarly at the same lipid dose.

- **Measurements of arterial blood pressure and heart rate**

About two weeks before micro-injection, an arterial catheter (model PA11-C40, Data Science) was implanted under tribromoethanol anesthesia (250 mg/Kg) into rat abdominal aorta. Arterial blood pressure (MAP) and heart rate (HR) were registered by telemetry during 10 seconds every 10 minutes for up to 12 days after microinjection in undisturbed freely-moving rats.

Results and discussion

- **Effects on arterial blood pressure and its circadian rhythm**

Micro-injection of LAng elicited a significant pressor effect during the day-time that was maintained for 5 days. This long-lasting pressor effect contrasts with the 15 min-pressor effect observed when the peptide is microinjected in the free form at the same dose. The highest blood pressure was obtained on day 3 (114 ± 4 mmHg) that differed significantly from that registered on day 0 (100 ± 3 mmHg). As expected, Lemp did not produced any significant pressor effect (94 ± 5 mmHg on day 3 vs 90 ± 5 mmHg on day 0). Night-time MAP, in contrast to day-time MAP, was not significantly affected by the micro-injection of LAng. These data show that liposomes act as an efficient sustained release system of Ang-(1-7) at the RVLM and established, in chronic conditions, the pressor effect of Ang-(1-7).

Under normal physiological conditions, blood presure and heart rate suffer circadian variation, with a higher level in the awake state and a lower level during the sleep period (corresponding to the day-time in rats). The pressor effect of LAng on day-time and its lack of effect on night-time resulted in the alteration of the normal circadian fluctuations of MAP. Strikingly, MAP on day 2 was found to be significantly higher during the day-time than in night-time (paired *t* test, $P < 0.05$). This apparent inversion of the circadian rhythm of MAP was confirmed by the mean acrophase determined on day 2 in LAng group (01:07 PM) that differed significantly from the mean acrophases found on day 0 (11:53 PM) and on day 2 in Lemp (10:42 PM) (Watson´s F tests, $P < 0.05$).

- **Effects on heart rate and its circadian rhythm**

Micro-injection of LAng also produced a significant decrease of HR on night-time, that was maintained for at least 3 days. The peak of bradycardia occurred on day 2 with a night-time value (319 ± 9) that differed significantly from that obtained on day 0 (360 ± 6). Such an effect was not observed on day-time or following micro-injection of Lemp.

The effect of LAng on night-time HR and its lack of effect on day-time HR also resulted in the alteration of the normal circadian variations of HR. Therefore, our data uncovers a new action of the peptide at the RVLM: modulation of HR and its circadian rhythm.

- **Study of liposome localization**

The localization of DiI-labelled liposomes was visualized under a fluorescent microscope in serial sections of the micro-injection area (24 hours and 7 days after micro-injection). This data strongly suggests that liposomes remained concentrated in the RVLM and were removed very slowly from the micro-injection site.

Conclusions

Two significant advances can be highlighted from our study. From the methodological point of view, it is the first time that the potential of liposomes as a sustained release system in a specific site of the brain is established. It is felt that this approach could turn out to be an important tool for the elucidation of the physiopathological role of neuro-modulating peptides in the brain. From the physiological point of view, this study established, in chronic conditions, the pressor effect of Ang-(1-7) at the level of the RVLM. Remarkably, it unmasked a new physiological role for Ang-(1-7) at the level of the RVLM: modulation of the circadian rhythms of blood pressure and heart rate.

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DD-09 SUBSTANTIVITY EVALUATION OF THE SUNSCREEN OCTYL METHOXYCINNAMATE IN DIFFERENT SYSTEMS: LIPOSOMES, LIPOSPHERES, β-CYCLODEXTRIN AND CHITOSAN COMPLEX

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Introduction

An ideal sunscreen can be classified according to its optical and physical characteristics. Optically, the ideal sunscreen should absorb light over a broad spectrum. Physically, the ideal agent should penetrate easily and adhere well to the skin, resist removal by sweat and particularly by water, without frequent reapplication[1]. Substantivity is the characteristic of a sunscreens that reflects how effectively the advertised degree of protection is maintained under adverse conditions, including repeated water exposure or sweating. Several approaches were studied to improve the protective efficiency of this formulation group. Natural and synthetic complex polymers used in addition of correct selection of solvent vehicle was studied to improve the performance of sunscreens [2]. At the same time, encapsulation process was investigated in order to enhance the water resistance and optical properties of these formulations [2].

The marine biopolymer chitosan due to its cationic character has high substantivity to the skin. Also due to the high molecular weight, it forms an elastic, and strongly adhesive and protective film [3].

The advantage of encapsulate sunscreens formulations in liposome, liposphere or cyclodextrins is to the enhance significantly the accumulation of sunscreen at site of administration as a result of the high substantivity with *stratum corneu*m [4-6].

Here in, we propose to investigation of new formulations using Chitosan, cyclodextrins, lipospheres and liposomes technology, looking for the production of new ideal sunscreen preparations. *in vitro* and *in vivo* evaluations will be performed.

Experimental methods

For cyclodextrins inclusion process, β-cyclodextrin was used in analytical grade; Octyl metoxycinnamate (OMC) were pharmaceutical grade. The inclusion formation was observed by the use of infrared and differential scanning calorimetry (DSC) analysis); UV analysis was used to measure the inclusion yield. The *in vitro* Sun Protection Factors (SPFs) of these sunscreens have been determined as described by Mansur et al. [7]. Absorbances of each preparations at a final concentration of 0.2µL/mL diluted in ethanol (in triplicate) have been determined through the use of UV-visible Spectrophotometer Shimadzu UV-1601. The *in vivo* SPFs have been determined as by FDA [8]. The subjects have been 20 human volunteers with skin types I, II and III, and solar ultraviolet simulators Multiport 601-Solar Light Company have been used. The water resistant property have been determined by evaluating the subject after two 20-min immersions with moderate activity in water. The liposomes and the lipospheres (with 4% OMC) were incorporated in hydroxyethylcellulose gels. The cyclodextrins and the chitosan (0.2%) complex (with 6% OMC) were incorporated in non-ionic emulsion. These preparations were compared with the controls gel and cream with 4% and 6% of OMC respectively. The standard was a lotion with 8% homosalate (Table1)

Results and discussion

The best inclusion results in β-cyclodextrin were obtained with the use of kneading inclusion technology. The DSC analysis showed a new endotherm related to the inclusion complex; however some amount of β-cyclodextrin was also detected in the mixture. A satisfactory inclusion yield was registered with this sunscreen, ca 60-70%.

Liposomes were prepared by the swelling of dry phosphatidylcholine films with OMC deposited in around-bottomed flask in buffer under gentle or vigorous shaking. The amount of phospholipid used was 280 µM/mL, and the percentage of OMC was 20% of total phospholipids [9].

Lipospheres were prepared utilizing the moisture tripalmitine:lecytine (1:1) as lipid matrix. The micro-dispersion system was characterized by analysis in polarized optic microscopy and phosphorus analysis. The entrapment yield was 65-70 % for OMC[10].

Table 1.
Values of *in vitro* and *in vivo* SPFs of the different systems with Octyl methoxycinnamate (OMC)

Sunscreen in different systems	SPF *in vitro*	SPF *in vivo*	SPF *in vivo**
Liposome gel (4% OMC)	7.6 ± 0.24	9.3 ± 0.9	7.3 ± 0.7
Lipospheres gel (4% OMC)	8.2 ± 0.42	9.7 ± 1.8	6.8 ± 0.71
Control gel (4% OMC)	7.4 ± 0.17	8.4 ± 0.9	6.3 ± 0.7
Chitosan cream (6% OMC)	10.5 ± 0.15	9.1 ± 1.4	6.5 ± 0.88
Cyclodextrin cream (6% OMC)	10.7 ± 0.19	9.0 ± 1.07	5.4 ± 0.76
Control cream (6% OMC)	11.2 ± 0.35	7.5 ± 0.8	5.8 ± 0.55
Standard lotion (8% homosalate)	4.5 ± 0.5	5.0 ± 0.1	–

*(after immersions)

Conclusion

The *in vivo* test performed with new sunscreens formulations showed that cyclodextrins complex had little effect in produce optical and water resistance. Liposomes, lipospheres and chitosan complex have changed the wash off and SPF values measured, with the best results related to the liposome formulations. However, due to its low coast and easy production we conclude that chitosan is a best choice for new sunscreen formulations development.

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DD-10 *IN VITRO* CUTANEOUS AND PERCUTANEOUS PENETRATION OF PAROMOMYCIN FROM LIPOSOMES

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Introduction

Paromomycin (PA), an aminoglycoside antibiotic, has been tested as an alternative topical treatment against cutaneous leishmaniasis (CL). Although this treatment has shown promising results it has not been successful in accelerating the recovery in most cases. This could be attributed to the low skin penetration of this hydrophilic antibiotic. Dermal delivery of hydrophilic drugs usually creates problems due to their inability to penetrate in the *stratum corneum* (SC) [1]. Liposomal formulations provide sustained and enhanced levels of these drugs in deeper strata of the skin, when compared with conventional preparations.

The objective of this work was to prepare liposomal formulations containing PA for the topical treatment of CL and to study their potential for to enhance the skin penetration of PA.

Experimental methods

- **Materials**

Paromomycin sulfate (PA), potency of 718 mg/g, (Pharmacia & Upjohn, Italy), soybean phosphatidilcholine (PC) and cholesterol (CH) were obtained from Lipoid (Germany). All other chemicals used for analysis were analytical reagent grade.

- **Preparation of liposomes containg PA**

Large unilamellar vesicles (LUV) containing PA were prepared by reverse-phase evaporation method. PC (30 mM) or PC:CH (30 mM, molar ratio 1:1) were dissolved in chloroform/methanol (2:1, v/v) in a round-bottomed flask. The solvent was then removed by vacuum evaporation using rotary evaporator. The lipid film was dissolved in 9 mL of diethyl ether. Next, 3 mL of PA solution (10 or 100 mg/mL) in phosphate buffer (pH 8) were incorporated. The mixture was sonicated for approximately 5 minutes. The resulting w/o emulsion was placed in a rotary evaporator for evaporation of the organic phase.

- **Determination of drug entrapment**

Encapsulated PA was separated from free by ultracentrifugation at 14,000 g for 60 minutes. The LUV were resuspended in phosphate buffer (pH 8) and a sample (100 μL) was added to equal volume of n-propanol [2], to disrupt the vesicles. The PA concentration was evaluated by microbiological assay [3].

- ***In vitro* percutaneous absorption**

The percutaneous absorption experiments were performed on skin biopsies of males hairless mice mounted over modified Franz diffusion cells (membrane surface area of 1.77 cm^2 and volume of the receptor compartment of 6.7 cm^3). The mice were sacrificed by cervical rupture and the dorsal skin was excised. The experiments were performed in the presence or absence of the SC. The SC was removed by stripping 15 times with a cellophane adhesive tape (3M Co., n° 810, USA). The receptor compartment was filled with 0.05 M phosphate buffered saline (pH 7.4) containing 0.01% of preservative ($HgCl_2$) and maintained at 37°C. After the equilibrium time (12 hours), the receptor fluid was removed and substituted for phosphate buffered saline without preservative.

100 μL of the preparations were applied on the skin using a Stepper 411 pippetor. The receptor fluid was removed in specific times and refilling with fresh solution.

After the last sampling, the preparations remained on the skin were recovered by washing the treated surface twice with 0.5 mL of solution of polyoxyethylene 20 oleyl ether (Sigma, EUA) 1% and 0.5 mL of water. Next, the skin surface was dried with a cotton swab. The epidermis and dermis were separated using a scalpel. PA extraction from dermis and epidermis was made in 5 mL of phosphate buffer (pH 8) using a Ultra-Turrax for to dilacerate the tissue. The PA concentration was determined by microbiological assay [3].

Results and discussion

The influence of the composition of liposomal preparations in the PA entrapped was investigated (Table 1). The PC LUV showed an entrapped PA content greater than that observed for liposomes of PC:CH. Besides, the increase of PA concentration in the hydrating solution increased the entrapment.

Table 1
Influence of lipid composition (PC and PC:CH at 30 mM) and PA concentration in the hydrating solution on entrapped PA content in LUVs liposomes.*

Lipid composition	% entrapment	
	10 mg/ml	100 mg/mL
PC	24.2 ± 4.4	41.9 ± 6.2
PC:CH	16.0 ± 1.3	27.2 ± 2.4

*Data are means (n=4) ±SD.

In presence of SC (Table 2), total recovery was of 87 to 95% of the applied dose and the amount of absorbed PA (receptor) was low independent of the tested formulation. However, the percutaneous absorption and dermal penetration (epidermis + dermis) of PA from LUVs liposomes (PC or PC:CH) were significantly higher (ANOVA; $p<0.05$) than those observed from empty LUV+PA and aqueous solution of the drug. Liposomes enhance percutaneous penetration of drugs through an interaction with SC and this may explain the higher drug concentrations in the skin [4].

Table 2
Skin distribution of PA *in vitro* (% applied dose) from liposomal formulations (100 mg/mL) across intact hairless mice skin.*

Formulations	Compartment				
	Receptor	Epidermis	Dermis	Surface	Recovery
PC LUV	1.6 ± 0.3	2.2 ± 0.1	0.7 ± 0.1	85.3 ± 7.2	89.7
PC:CH LUV	1.3 ± 0.4	3.0 ± 0.2	0.6 ± 0.2	89.6 ± 11.7	94.6
Empty LUV+PA	0.2 ± 0.1	0.7 ± 0.01	0.4 ± 0.04	90.6 ± 7.9	91.9
PA solution	0.5 ± 0.2	0.5 ± 0.04	0.2 ± 0.03	85.9 ± 2.4	87.1

*Data are means (n=3) ±SD.

Surprisingly, in absence of SC (Table 3), the amount of PA absorbed from liposomes (PC or PC:CH) was also greater than that observed from solution or empty LUV plus PA. In fact, it could be attributed to application of liposomal suspensions containing free and entrapped drug.

Conclusions

The entrapment of PA in PC or PC:CH LUV increased when the PA concentration in the hydrating solution increased.

Table 3
Skin distribution of PA *in vitro* (% applied dose) from liposomal formulations (10 mg/mL) across hairless mice skin in absence of SC.*

Formulations	Compartment				
	Receptor	Epidermis	Dermis	Surface	Recovery
PC LUV	91.0 ± 2.8	5.3 ± 0.5	11.6 ± 1.8	3.5 ± 0.4	111.3
PC:CH LUV	91.5 ± 3.7	3.9 ± 0.5	12.5 ± 1.2	2.2 ± 0.2	110.1
Empty LUV+PA	71.6 ± 7.1	6.1 ± 1.3	16.2 ± 3.0	7.5 ± 0.9	101.3
PA solution	74.9 ± 2.3	6.0 ± 0.4	9.4 ± 2.2	6.8 ± 0.4	97.1

*Data are means (n=3) ±SD.

The percutaneous absorption and skin penetration of entrapped PA was greater than that of PA in solution and empty LUV+PA. Topical delivery of PA was increased by entrapment in LUVs liposomes.

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DD-11 DEBITTERING OF CASEIN HYDROLYSATES BY ENCAPSULATION IN LIPOSPHERES

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Introduction

The interest in introducing protein hydrolysates in food has been increased. These hydrolysates can be useful for people showing intolerance to proteins or enzymatic deficiency [1]. However, the inconvenient related to the protein hydrolysates is their unpleasant taste produced during the enzymatic hydrolysis. The bitter taste observed is due to the release of hydrophobic peptides in the course of proteolysis [2]. The use of the encapsulation technology can be a strategy to mask this taste, contributing to increase the acceptance of consumers to this kind of food product. Therefore, in this work, we have studied the encapsulation of casein hydrolysates in lipospheres.

Experimental Methods

- **Materials**

Bovine casein and anilinonaphthalene sulphonate were obtained from Sigma Chemical Company (St. Louis, MO, USA). Soybean lecithin was gently supplied by Lipoid GmBh (Darmstad, Germany). Papain was a gift of Biobrás (Montes Claros, Brazil). Thiobarbituric acid was purchased from Merck (Darmstad, Germany). Stearic acid was obtained by Galena Química e Farmacêutica Ltda (Campinas, Brazil).

- **Casein Hydrolysates Preparation**

Three casein hydrolysates were obtained by using different proteolysis conditions. The first hydrolysate (H1) was prepared using an enzyme/substrate percentual ratio of 2.0, at 37°C, pH 7.5, during 5h of incubation. The second (H2) and third (H3) hydrolysates were also prepared at pH 7.5, under 5h of incubation, employing an enzyme/substrate percentual ratio of 4.0. The temperature of incubation was 40 and 60°C for H2 and H3, respectively.

- **Lipospheres Preparation**

Lipospheres encapsulated casein hydroysates were prepared by melt process [3]. Briefly, 320mg of each casein hydrolysate were dispersed in 630mg of melted stearic acid. Then, a hot 0.1M phosphate buffer solution containing soybean lecitihin (1%) was added. The mixture obtained was homogenized for about 3min., at 9,500rpm, using a homogenizer (Ultra-Turrax IKA Labortechnik, IKA T25 Basic Model, Germany). The milky formulation was then rapidly cooled down to about 20°C by immersing the formulation flask in an acetone-ice bath while homogeneization was continued. Finally, the lipospheres obtained were lyophilized and maintained at 4°C. Non-entrapped casein hydrolysate was separated by centrifugation (3 times, 14,000 rpm, 90min.) for the determination of the casein hydrolysate entrapment percentage in lipospheres. This determination was made by second derivative spectrophotometry developed by Silvestre *et al.* [4]. The morphology and particle size of lipospheres were determined by scanning electron microscopy. The stability of lipospheres was evaluated by measuring of lipidic oxidation along the time (0, 15, 30, 45 and 60 days). This measurement was made by the determination of thiobarbituric acid reactive substances as described by Rosmini *et al* [6].

Hydrophobicity Determination

Measureaments of the hydrophobicity of lipospheres containing or not casein hydrolysate and of the pure casein hydrolysate (not submitted to the encapsulation process) were performed as described by Boatright & Hettiarachchy [5].

Sensory Evaluation

Bitterness intensity was evaluated by six trained panelists on a 6-point scale, as previously described [6].

Results and Discussion

The casein hydrolysate entrapment percentage in lipospheres was 50, 65 and 83% for H1, H2 and H3, respectively. Microscopic examination of the formulation containing H2 showed spherical particles with an average size of 3.8µm. The evaluation of lipidic oxidation of lipospheres containing H1, H2 or H3 showed no alteration in the period of time up to 60 days. These findings indicate a good stability of lipospheres, which can contribute to the retention of entrapped casein hydrolysate.

The encapsulation of the three casein hydrolysates in lipospheres led to a 52, 53 and 36% reduction of the hidrophobicity reduction for H1, H2 and H3, respectively. Among the three lipospheres studied that one containing H3 showed the largest hidrophobicity decrease which can, probably be associated to its higher entrapment into lipospheres (83.0%).

Finally, the encapsulation of casein hydrolysates in lipospheres reduced their bitter taste, as shown in Figure 1. Bitterness scores of H1, H2 and H3 encapsulated in lipospheres decreased of 18, 33 and 30%, respectively. In fact, the observed decrease of hidrophobicity is related to the bitter taste masking.

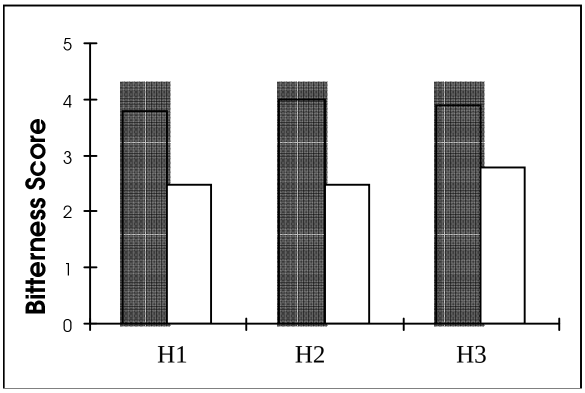

Figure 1 - Sensory evaluation of casein hydrolysates H1, H2 and H3 before (▨) and after encapsulation in lipospheres (☐).

Conclusion

The present study showed that casein hydrolysates can be efficiently encapsulated in lipospheres. Furthermore, the encapsulation of casein hydrolysates produced a decrease of their hydrophobicity, and consequently, contributed to mask their bitter taste.

Acknowledgements

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DD-12 SCALLING UP OF LIPOSOMES PRODUCTION BY SPRAY DRYING METHOD: INFLUENCE OF OPERATIONAL VARIABLES ON THE CHARACTERISTICS OF DRIED PARTICLES AND LIPOSOMES PRODUCED

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Introduction

The spray drying method for a mass production of a dried lipid mixture is an alternative technique for the scalling up of liposomes production [1,2,3]. Although the technological importance of this technique, engineering studies still are scarce in this area. Liposomes produced by this method are multilamellar vesicles obtained by hydration of dried lipid particles. During the drying process, lipid molecules aggregate producing dried membranes. The cristalinity degree is an important property of these membranes which influences the hydration performance of liposomes and consequently the entrapment and delivery of drugs [1].

The aim of this work was the investigation of the influence of operational variables in the spray drying process on the characteristics of dried particles and liposomes.

Experimental Methods

- **Materials**

Soybean phosphatidylcholine, Epikuron 200 SH (Lucas Meyer), mannitol, ethanol and sodium chloride (Synth), and Hepes buffer (Sigma).

- **Preparation of dried lipid particles by spray drying method and characterization**

The spray drying products were prepared by a laboratory type spray drying, a Lab Plant spray model SD-04. In the preliminary experiments the lipid in a molar ratio of 20mM was dissolved in 250mL of ethanol, with or without mannitol, used as a core material, in a molar ratio of 3:2 (lipid:mannitol). The organic solution of lipid was sprayed into a drying chamber through a spray nozzle whose orifice size was 1mm and with an inlet flow rate of 3,5mL/min. The inlet temperature of the chamber was 90^0C. The spray drying product was essentially obtained from the reservoir attached to the cyclone and the dried membranes was stored in refrigerator.

The relative importance of the operational variables on the mass production was analysed by a statistical fractionary fatorial design 2^{4-1} with three central points for the error. The variables investigated were the drying temperature (T) (90, 100 and 110^0C), lipid concentration in the initial dispersion (C) (15, 30 and 45mM), the liquid flow rate (F) (3, 14 and 30 mL/min) and the presence of mannitol in the initial dispersion (M) (0, 15 and 30%). The results were analysed in terms of the dried lipid mass yield. The dried particles were characterized in terms of the size distribution by laser light scattering measurements (LA-900 Laser Scattering Particle Size Distribution Analyser, from Horiba Instruments, Inc.), and membrane cristalinity by X-rays diffraction (Philips X-rays, model XPERT).

- **Liposomes production and characterization**

Liposomes were produced by hydration of the dried lipid particles with a Hepes/NaCl (10mM/120mM) buffer solution. The mean vesicle size and polydispersity index (PI) determined by QELS measurements using a Malven Autosizer, model 4700.

Results and Discussion

The experiments showed that dried lipid particles containing mannitol are more amorphous compared with that ones without mannitol (Figure 1).

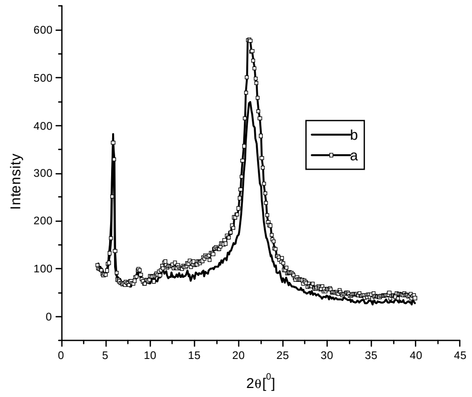

Figure 1: X-rays of dried lipid particles (a) with mannitol (b) without mannitol.

The statistical contrasts of the operational variables on the various levels, calculated from the fractionary fatorial design, showed that the lipid concentration in the initial dispersion and the liquid flow rate are the more significant operational variables on the mass lipid yield in spray drying process (Figure 2).

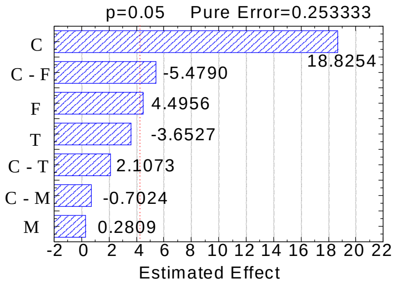

Figure 2: Pareto’s figure from the fractionary design showing contrasts of the operational variables and their interactions related on the massic lipid yield.

The dried particles mean size was 9±1.5µm with a distribution mainly between 0.75 and 40µm for all the preparations, except for the extreme levels of flow rate and lipid concentration, where particles were larger ranging from 10 to 150µm. Multilamellar liposomes were produced from dried particles, independently of the operational level used on the spray drying process. The size distribution ranged from 0.7 to 1.5µm with mean diameter of 1.1µm for the vesicles without mannitol, and from 1 to 3.5µm with mean diameter of 2µm for the vesicles with mannitol. Both vesicles presented a high polidispersity, how it was expected from multilamellar vesicles.

Conclusions

These results indicate that the spray drying is a promising technique for scalling up of the liposomes production. Lipid concentration and flow rate are the more significant variables on the yield of the process. The cristalinity of the lipid particles can de controlled by the mannitol concentration, potentializing the modulation of their capacity for incorporation and delivery of drugs.

Acknowledgements

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DD-13 EFFECT OF NMF LIPOSOMES IN THE *STRATUM CORNEUM* COHESION

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Introduction

The main function of the skin is to protect the body against exogenous substances. The skin barrier is located in the outermost layer of the skin, the *stratum corneum* (*sc*). This layer consists of keratin enriched dead cells surrounded by crystalline intercellular lipid domains [1].

These lipids form multilamellar sheets within the intercellular spaces of the *sc*. This organization is essential in maintaining the functionality of the skin as an effective barrier to water loss. It also plays an important role in determining the mechanical, cohesive and desquamatory properties of the *sc* [2].

Several data has shown that the liposomes have a marked influence in the penetration of labeled drugs [3, 4 and 5]. Hofland and co-workers [6] using the freeze fracture technique showed ultra-structural changes in the *sc* of the human skin, after incubation with liposomes. The constituents of the liposomes, moleculary dispersed into the *sc*, mix with the *sc* lipids and induce a penetration-enhancing effect by producing ultrastructural changes in the intercellular lamella.

The objective of this work was to quantify the *sc* cohesion in function of the volunteers' ages and after a 20-sereal-day treatment with NMF liposomes.

Experimental methods

- **Population**

Thirty volunteers were tested free of active skin diseases in the test area and separated into three age groups, from 20 to 30, from 30 to 40 and from 40+ years.

- **Chemicals**

The following solutions and reagents were utilized: NaOH, HCl, deionized water, alkaline copper tartrate solution, dilute Folin reagent, Pro-Lipo H (Lucas Meyer, USA), NMF (Natural Moisturizing Factor).

- **Adhesive tape**

As adhesive tape, D-Squame disks (CuDerm Corporation, USA) with a diameter of 2.2 cm (area 3.80 cm^2) were used.

- **Preparation of liposomes**

Liposomes were prepared using the pro-liposome method. An aqueous dispersion of 10 w% Pro-Lipo H containing 0.25 w% NMF was prepared under stirring for 15 min at 65°C followed by sonnication for 15 min. The obtained liposomes were characterized by dynamic light scattering on a Zetasizer (Malvern Inst., U.K.). The diameter of the NMF containing liposomes was 125 nm; the polydispersity was 0,331. Entrapment efficiency was 80% under these conditions.

- **Preparation of a NMF liposomes gel**

NMF liposomes were added to a cellulose hydroxyethyl solution (1.5%).

- **Sequential tape stripping using disks**

Tape stripping was performed on the volar forearm in the center between the cubital fossa and the wrist of the thirty volunteers. The marked area on the skin of the volunteers was sequentially stripped 10 times after applying constant and uniform pressure for 10 seconds. After gentle removal from the skin, the disks were placed into glass vials containing 1 ml 1 M NaOH. Neutralization was performed after shaking the vials for 2 h.

- **Protein assay**

The Lowry's modified method was used for the protein assay. The method is based on the quantification of the sodium hydroxide soluble protein fraction. The tape stripping technique and the protein assay were realized before and after the 20-serial-day treatment with the NMF liposomes.

- **Qualitative analysis**

Two other adhesive disks were used for qualitative analysis. They were stained with toluidine blue and basic fuchsin. Analysis was made through optical microscope, following established approaches.

Results

We collected samples of a desquamating *stratum corneum* from the volar forearms of adults, aged 20-65.

Before the treatment with the NMF liposomes, we observed some changes related to the age group.
The 20-30 group showed homogeneous results in the total *sc* levels (Figure 1). In normal skin, only the outermost three to four cell layers were removed, the lower layers were firmly bonded.

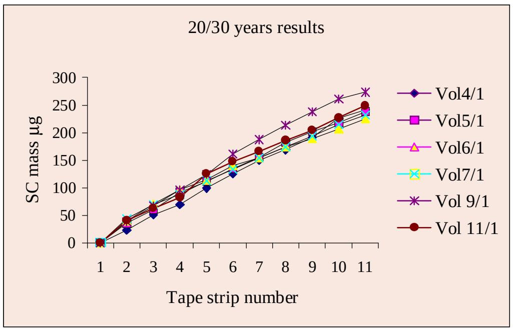

Figure 1. *Sc* removed by sequential tape stripping for six human volunteers

This is not the case with age, where intercellular cohesion is unevenly altered with the accumulation of corneocyte clumps. The whole upper *sc* displays an altered cohesiveness, and the desquamation scales are much thicker, though not uniform.
After a 20-serial-day treatment with NMF liposomes, the *sc* cohesion and skin moisturizing was improved in all volunteers independent of the age group (Table I).

Table I. Accumulated *sc* removed by sequential tape stripping on six volunteers of each group on the first day (D1) and 20 days later (D21).

Age	Volunteer	Total *SC* mass (µg/cm2) D 1	Total *SC* mass (µg/cm2) D 21
20/30	3	193	135
	6	225	184
	7	231	163
	9	273	199
	11	248	202
	12	303	162
30/40	13	191	135
	15	243	72
	16	149	34
	18	178	98
	20	197	78
	21	146	80
>40	23	287	87
	25	164	27
	26	227	194
	27	197	79
	30	278	80
	31	175	99

We found a significant correlation among the microscopical assessment of the *sc* cohesion and protein assay. All the volunteers demonstrated in the microscopic degree the effects of moisturizing and the best cohesion after using NMF liposomes.

Conclusion

The results demonstrated that the tape stripping technique and protein quantifying method can be used for the *sc* cohesion evaluation. This study has shown the influences of age and liposomes used in three volunteer groups. Others studies can be done to investigate the differences in the *sc* composition among ethnic, distinct worker, gender and dissimilar sun exposition groups.

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DD-14 PREPARATION AND EVALUATION OF DRUG-DELIVERY SYSTEMS FOR MEPIVACAINE AND BUPIVACAINE

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Introduction

Local anesthetics (LA) are a class of chemicals that reversibly block peripheral and central nerve pathways following regional administration. They can inhibit sensory and motor impulses causing pain relief and justifying their clinical use. Ideal LA molecules must have long action, low toxicity and selectivity for sensory than for motor block [1].

The development of LA formulations in carriers such as liposomes and cyclodextrins offer the possibility to control drug-delivery in biological systems, by prolonging the anesthetic effect and/or reducing their toxicity [2].

This work aims to compare the motor block and the antinociceptive activity, *in vivo*, of two drug-delivery systems of LA, in relation to their commercial solutions.

The anesthetics chosen (Mepivacaine and Bupivacaine) belong to the amino-amide family.

- **Materials**

Bupivacaine (BVC) was a gift from Cristalia Ind. Quim. Farm. Ltda. Mepivacaine (MVC), egg phosphatidyl choline (EPC), cholesterol (CHOL), α-tocopherol (α-TC) and β-cyclodextrin (β-CD) were purchased from Sigma Chem. Co.

- **Sample preparation**

0.4μm unilamellar vesicles (LUV, EPC:CHOL:α-TC, 4:3:0.07, mole fraction) were prepared by extrusion, from multilamellar vesicles . BVC or MVC was added after vesicle formation, in order to obtain the same concentration of the plain solutions (0.5% or 15.4mM). $BVC_{\beta\text{-CD}}$ or $MVC_{\beta\text{-CD}}$ (1:1 molar ratio) inclusion complexes were prepared by shaking the LA and β-CD in aqueous solution. After equilibrium, the solution was freeze-dried and stored at – 20 ^{o}C.

- **Biological tests**

Male Swiss albino mice (weighting 30-35 g) were purchased from CEMIB-Unicamp.

Plain, liposomal or βCD-complexed preparations of BVC or MVC were injected (0.1mL) by infiltration into the right posterior limb of the mice. LA activity was evaluated by the loss of motor control in the injected limb, with scores ranging from 0 to 2 (normal movement to paralysis). The evaluation was done from 1 – 5 min. and thereafter every 10 min. up to 1 hour following the injection (n=8) [3].

The antinociceptive activity was evaluated by the paw pressure test (Ugo Basile Analgesymeter), with a maximum pressure of 150g, to avoid paw injury (n=10) [4].

- **Statistical analysis**

Analysis of variance (ANOVA) with the *post hoc* Tukey-Kramer test were used for comparison between the groups.

Results and discussion

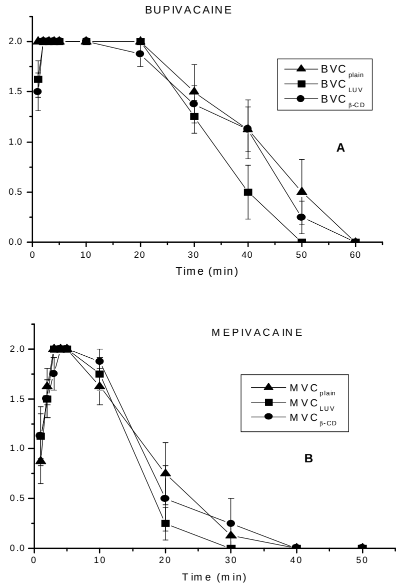

Figure 1. Scores (Mean±sem) for BVC (A) and MVC (B) motor block action with plain solutions, liposomal or β-CD formulations; [LA]= 15.4 mM (n=8).

Figure 1 shows that motor function is affected by the injection of both BVC and MVC in the region of the sciatic nerve. Nevertheless the overall motor function was not significantly different between plain, liposomal or β-CD complexed formulations.

These results are summarized in Table 1, that compares the amplitude and the duration of the motor block. As expected, BVC presented higher potency than MVC but not significant differences were registered between the drug-delivery systems for any of the anesthetics.

Table 1: Time for recovery (mean±sem, min.) and total effect, estimated by the area under the curve (AUC). No significant changes (ANOVA) were found between the groups.

GROUPS	MOTOR BLOCK DURATION (*min*)	AUC (*score/h*)
BVC_{plain}	47.5 ± 3.5	79.2 ± 6.9
BVC_{LUV}	43.5 ± 2.0	65.3 ± 4.1
$BVC_{\beta\text{-}CD}$	51.0 ± 2.0	74.0 ± 5.6
MVC_{plain}	26.0 ± 2.5	33.0 ± 4.7
MVC_{LUV}	22.5 ± 1.5	27.7 ± 2.5
$MVC_{\beta\text{-}CD}$	23.5 ± 2.5	33.4 ± 5.4

In Figure 2, paw pressure tests show that infiltration of the vehicles (LUV or β-CD) didn't modify the baseline of the animal's answer. Once again the longer anesthetic effect of BVC over MVC was registered. Statistical tests revealed differences in the amplitude and duration of the antinociceptive effect of both liposomal and β-CD:complexed formulations, in relation to the plain solution of BVC and MVC.

For BVC, the group treated with the β-CD:complex showed the high increase in the mean answer to pressure, when compared to BVC_{plain} or BVC_{LUV} (*$p<0.001$*).

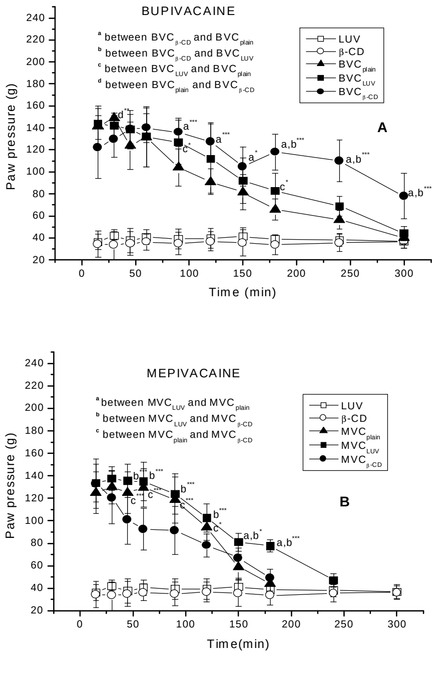

Figure 2. Time course (min.) of paw pressure tests with vehicles (LUV and β-CD), BVC_{plain}, BVC_{LUV} and $BVC_{\beta\text{-}CD}$ formulations (A) and MVC_{plain}, MVC_{LUV} and $MVC_{\beta\text{-}CD}$ (B). Values were expressed as mean±sd (n=10) **p<0.05, **p<0.01, ***p<0.001.*

By the other hand, MVC_{LUV} was the more efficient formulation for mepivacaine, increasing and prolonging the duration of analgesia - up to 240 min. after injection - when compared to MVC_{plain} and $MVC_{\beta\text{-}CD}$ (*$p<0.001$*).

Conclusions

This work shows the relation between motor and sensory block, induced by BVC and MVC drug-delivery formulations. While none of the formulations were able to modify the motor block duration, $BVC_{\beta\text{-}CD}$ and MVC_{LUV} formulations improved the antinociceptive effects (amplitude and duration) of the LA, relatively to the plain solutions.

We believe that the low solubility of BVC and low membrane partition of MVC, relatively to other clinically used LA, were improved by the encapsulation systems, respectively, increasing their bioavailability and potency.

As for the longer anesthetic effect shown here, one has to consider that clearance is decreased both by complexation of BVC inside β-CD cavity and by MVC encapsulation inside the liposomes.

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DD-15 INCORPORATION OF PRAZIQUANTEL INTO PHOSPHATIDYLCHOLINE CONTAINING-DISPERSION

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Introduction

Praziquantel (PRZ) is the drug of choice for treatment of schistosomiasis. However, it has been observed failure in mass treatment. This can be due to a low bioavaibility presented by PRZ [1]. The problem of bioavaibility can be caused by the low hydrosolubility of the drug that can difficult the dissolution and by the fast metabolization [2].

Recently, in our group, we verified that the incorporation of PRZ in liposomes of phosphatidylcholine (PC) can improve the hydrosolubility. However it was observed a very low entrapment efficiency [3].

It was observed in an *in vitro* study, using culture of *S. mansoni*, an effectiveness of samples of PC-liposomes with PRZ incorporated. So it is possible to maintain the effectiveness of PRZ in spite of the more hidrophilic medium. Moreover, the *in vivo* study had demonstrated an increase of effectiveness of PRZ in liposomes compared with PRZ in suspension [3]. So the development of a PRZ containing aqueous dispersion can improve the bioavaibility as well as possibility the administration by other routes, such as the parenteral route.

Considering the high effectiveness *in vivo* of these systems, it was also aim of this work to study of the ratio of PRZ, lipophilic drug, PC, as a solubilizant agent, and water to obtain homogeneous liquid dispersion as a preliminary study of a formulation with PRZ to try to improve the bioavailbility. So the aim of this work was to study some parameter that could affect the incorporation of PRZ into liposomes.

Experimental methods

- **Materials**

Chloroform and ethanol PA grade from Merck S.A. Phosphatidylcholine (Epikuron) from Luca Meyer. Cholesterol. Praziquantel from Merck. Tris (hydroximetil) aminemethano from Sigma.

- **Preparation of liposomes**

Liposomes were prepared from a chloroform solution of PC and CHOL. The solvent was evaporated under nitrogen flow and the dried-film was maintained in vacuum for 40 minutes. The dried-film was hydrated in 20 mM Tris/HCl buffer pH 7.5 and let 45 min in ice bath. The dispersion was then sonicated. The kinetic of vesicles formation was monitored by light scattering at 410nm.

- **Effect of temperature and composition of liposomes on incorporation of the PRZ**

Liposomes with several concentrations of PC with or without CHOL were mixed with 10 mg of PRZ. These dispersions were shaked during 30s and left to rest 1h in ice bath (0^oC), at room temperature (25^o) and at 37^oC. These dispersions were centrifuged and analyzed at 262 and 280 nm. The amount of PRZ incorporated was calculated by multicomponent spectrophotometry as standardized in work of our group [3].

- **Analysis of ratio of PRZ and PC to obtain liquid dispersion**

It was prepared a film containing different concentrations of PRZ and PC. This film was dried under N_2 flow and kept on vacuum by 2 hours. It was added different amount of water to obtain 5g of preparation and agitated in vortex until separation of film from the glass.

The dispersions was analyzed as a uniformity and as a dispersibility.

Results

The amounts of PRZ incorporated in liposomes of PC and PC:CHOL are showed in figure 1. The incorporation of PRZ in PC-liposomes increased at higher PC concentrations. In the presence of liposomes composed by PC:CHOL at 2:1 molar ratio, the incorporation of PRZ showed a small decreasing at PC concentrations of 240 and 280 mM.

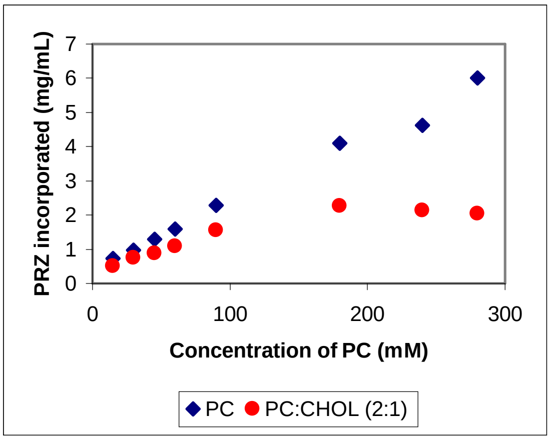

Figure 1: Amount of PRZ (mg/mL) incorporated in external layer in relation to PC concentration (mM).

The incorporation of PRZ into PC:CHOL-liposomes was smaller than that in liposomes without CHOL.

PC-containing liposomes (240 mM) with or without CHOL were used in order to verify the influence of temperature on the incorporation of PRZ. Results are showed in figure 2. The incorporation rate of PRZ increases as the temperature arises.

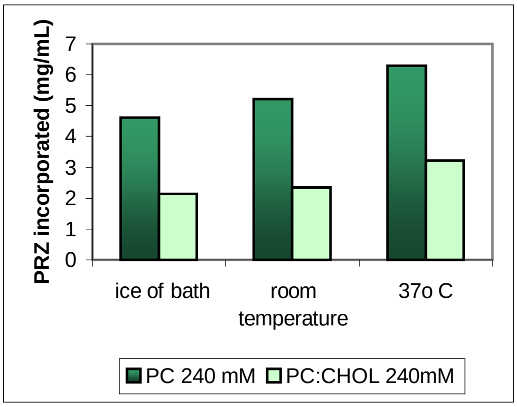

Figure 2: Effect of the temperature on PRZ incorporated in PC-containing liposomes and PC:CHOL- containing liposomes (240 mM).

In order to verified the PRZ/PC ratio required to obtain liquid dispersion, samples were prepared at different concentrations of PRZ and PC. It is necessary to use at least 33% (p/p) of water. Using 50% (p/p) of water, the maximum concentration of PRZ which can be incorporate was 40% (p/p) of the total mass of dispersion. However it is also possible to obtain good dispersion using 5% (p/p) of PC but diluted in 60% (p/p) of water (figure 3 and table 1).

Table 1: Dispersions with PRZ and PC in water

PRZ	PC	WATER	
25%	50%	25%	undispersible
33%	33%	33%	undispersible
50%	25%	25%	undispersible
10%	40%	50%	dispersible
10%	30%	60%	dispersible
20%	30%	50%	dispersible
20%	20%	60%	dispersible
30%	20%	50%	dispersible
30%	10%	60%	dispersible
35%	5%	60%	dispersible
40%	10%	50%	dispersible

Conclusions

These results showed that PC-containing liposomes improve the incorporation of PRZ in aqueous medium maybe by a possibility of the drug intercalate itself between the phospholipids of liposomes. On the other hand, the inclusion of CHOL in PC-liposomes can difficult this kind of incorporation and the temperature can improve it. This results demonstrated that is possible to prepare stable dispersions of PRZ in aqueous systems using a low concentration of the PC.

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Study *in vitro* and *in vivo* of praziquantel incorporated into phosphatidylcholine-containing liposomes
Presented in PCA – AAPS, Orlando, Florida

DD-16 CHARACTERIZATION OF LIPOSOMAL FORMULATIONS FOR DRUG DELIVERY

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Introduction

Liposomal drug-delivery systems have come of age in recent years, with several liposomal drugs currently in advanced clinical trials or already on the market. It is clear from numerous pre-clinical and clinical studies that drugs such as antitumor drugs, packaged in liposomes exhibit reduced toxicities, while retaining, or gaining enhanced, efficacy. This, results in part from altered pharmacokinetics, which lead to drug accumulation at disease sites, such as tumors, and reduced distribution to sensitive tissues. Another area of intense research is the application of liposomes exhibiting improved adjuvancy and stability (ganglioside mediated) for vaccine development. Rotavirus epidemiological incidence in Argentina and the rest of the world is high, during a season per year. The objective is to encapsulate protein fractions from rotavirus in sterically stabilized liposomes with long circulating times *in vitro*. We propose a strategy for liposomal formulation and characterization, using BSA (Bovine Serum Albumin) as a model solute.

Materials and Methods

The phospholipids and sphingolipids were from Avanti Polar Lipids: 1,2-bis (10,12-tricosadiynoyl)-sn-glycero-3 phosphocho-line), Bovine Brain Sphingomyelin; Gangliosides from Sigma, Bovine Brain GM Type III (20% n-acetyl-neuraminic acid) and other lipids from Lipoid: 1,2-dimiristoyl-sn-glycero- 3-phosphocholine (DMPC). Cholesterol was from Riedel de Haen and Bovine Serum Albumin (BSA) from Sigma.

- **Liposome preparation**

Multilamellar liposomes were prepared by dispersing a dry film of 40µmol total lipid in 800µl of 1mgr/ml BSA solution in Tris-HCl 10 mM buffer, pH 7.4. Diacetylenic lipid and DMPC (acting as spacer lipid) were dissolved in chloroform. They were mixed together in the desired mole ratio, typically 1:1. The solvent was removed under vacuum and further removed by leaving the mixture under a gentle stream of nitrogen. Lipids were resuspended in the same solution cited above. The liposomal solution so obtained was kept at least 1 hour at 30-35°C.

LUVs of c.a. 0.2 µm were obtained passing the liposomal solution, at least 13 times, through a Miniextruder (Avanti Polar Lipids) containing a filter membrane (0.2 µm), at 40-50°C.

BSA-containing vesicles were separated from free protein chromatographically, using a Sephadex G-200 column (1×16cm) with Tris-HCl buffer, at 20°C.

In the case of diacetylenic/DMPC mixture, the elute was polimerised at 4ºC in quartz cuvettes irradiated with UV-light (254 nm). Each cycle, of a total of 20, was set at 360,000 $\mu J/cm^2$ in a “cross-linking” Stratalinker 1800, during 3300 sec. The polymer formation was followed by visible spectral measurement (λ= 480-520nm).

- **Protein determination**

The concentration of encapsulated BSA was determined by subjecting liposomes (10, 15, 20µl) to SDS-PAGE electrophoresis along with known amounts of free BSA (0.5, 1, 2.5, 5µg) and visualising the protein by Coomasie Blue staining. When analysing the polymerised samples the method of choice for protein quantification was BCA method (Smith et al. [5]). SDS-PAGE gel electrophoresis was used to determine the concentration of the encapsulated protein. The density of the bands was determined by gel scanning and densitometry analysis (Sigma Gel software).

- **Lipid determination**

Phospholipid concentrations were determined using the phosphorous assay by reaction with Fiske-Subbarow reagent [4].

- **Stability measurement**

Fluorescent method: 10 ul of liposomal sample (50mM calcein encapsulated liposomes) was incubated with 2ml of buffer or serum at desired temperature. Fluorescence readings (λex.490nm, λem.520nm) were recorded during the first 3 hours, and a final measurement at 24 hours. Alternative method was to encapsulate glucose-6-phosphate and sugar released was measured enzymatically with glucose-6-phosphate-deshydro-genase(GPDH), at λ=340nm (Worthington Manual).

Results and discussion

The results obtained for proteins and quantitative determination of phosphorus for each formulation are depicted in Table 1.

Table 1. Lipid/Protein mass ratio for the different liposome formulations.

Liposome Formulation	[Lipid]$_T$ g/ml	[Protein] g/ml	Lipid/Protein Mass ratio
[EggPC/Chol] 1:1 mol ratio	0.0125	4x10^{-5}	286.5
[EggPC/Chol/SM] 1:1:1 mol ratio	0.0120	1.25x10^{-5}	961.0
[EggPC/Chol/SM/GM1] 1:1:1:0.14 mol ratio	0.0121	2.2x10^{-5}	553.0
[DIAPC/DMPC] 1:1 polymerised	0.0123	9.0x10^{-6}	1366.6

In the presence of buffer at 25°C EggPC/Chol vesicles shows a 24% lysis after 1 hour and stays stable up to three hours around the same value. EggPC/Chol/SM 1:1:1 mol ratio show a 15% initially and a 20% after three hours. EggPC/Chol/SM/GM 1:1:1:0.14 mol ratio reaches a 17% after 1 hour and c.a. 30% after 3 hours. Previous results with DMPC:DIAPC liposomes lysis measurements showed an augmented hydrophobic surface when polymerised. DIAPC/DMPC 1:1 polymerised showed no changes, but we have to consider that UV-irradiation might have affected calcein, Incubation in serum at 37°C gave considerable upper values: EggPC/Chol sample reached a lysis percentage of 38% after 3 hours, and so did EggPC/Chol/SM vesicles. Enzimatically the value is around 40%. In the case of EggPC/Chol/SM/GM the lysis was above 40% measured enzimatically after 3 hours of incubation. Calcein binds to serum proteins, this may complicate the interpretation of data obtained from in vivo or in vitro studies using this compound as a fluorescent marker, that is why we chose an alternative method. Both methods have the uncertainty of the polymerisation time (between 1h30min - 2 hours) during which the encapsulated material can leave the liposome. When we analysed the polymerised system with BSA using SDS PAGE we found cross-linked macromolecules after irradiation.

Conclusions

This study has shown that two main features for liposomes formulations involved in drug delivery systems, i.e. protein/lipid ratio (>200) and stability for long circulating moieties are preserved during a the elapsed time (3 hours) on binding to serum proteins. This formulations are going to be tested in the near future with rotavirus protein fractions (VP6, VP7 and VP4). The EggPC/Chol/SM/GM and the novel polymerised DIAPC/DMPC are the formulations to be tested in animal models, subcutaneous and orally. The latter one was chosen due to its high lipid/protein ratio and its cross-linking property which would enhance its antigenic adjuvancy.

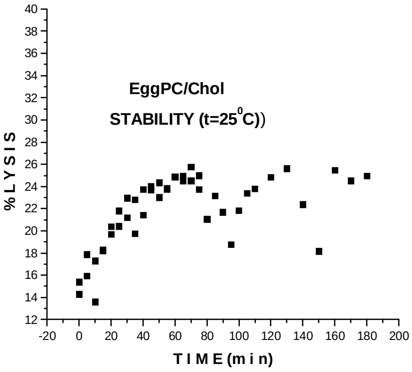

Figure 1. Typical stability representation of lysis percentage as a function of time. 100% corresponds to data obtained with TritonX-100 liposome rupture.

Acknowledgements

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DD-17 INFLUENCE OF THE DELIVERY SYSTEM (*STRATUM CORNEUM* LIPIDS LIPOSOMES) ON THE PARTITION COEFFICIENTS OF 5-AMINOLEVULINIC ACID (5-ALA) AND DERIVATIVES

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Introduction

Photodynamic therapy (PDT) is used to treat a wide variety of diseases involving the skin and other organs that can be reached by light. The therapy consists of the administration of a photosensitizer to the afflicted tissue, followed by a dose of light. The combination of photosensitizer and light produces singlet oxygen that in turn causes damage to the tissue. After topical administration, 5-ALA is enzymatically converted into protoporphyrin IX (PpIX), the precursor of heme in cellular heme synthesis and a clinically useful photosensitizer [1].

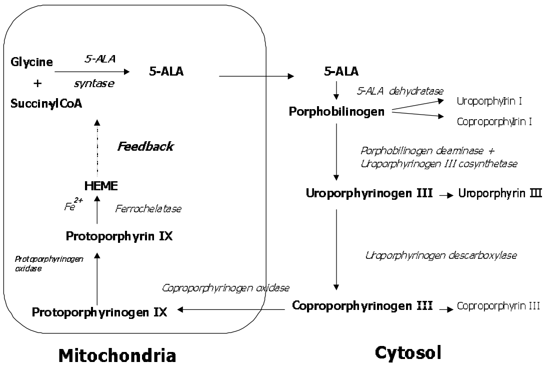

Figure 1: The heme biosynthetic pathway. The synthesis of 5-ALA occurs partly in the mitochondria and partly in the cytosol [2].

However, because of its low lipophilicity, 5-ALA also has a poor ability to diffuse through biological membranes such as the walls of stomach and intestine, the *stratum corneum* (SC) of the skin and cellular membranes. A possible solution to this problem is the use of ester derivatives of 5-ALA. These prodrugs are expected to have better diffusing properties as a result of their enhanced lipophilicity and are converted into the parent ALA [3]. *Stratum corneum* lipids liposomes (SCLLs) have been proposed as skin drug localizers, improving in this way the biodistribution and consequently the local concentration of the encapsulated drug [4].

Purposes

The main purpose of this work was to determine the partition coefficients: The octanol/water ($K_{O/W}$), skin/water ($K_{S/W}$) and skin/vehicle ($K_{S/V}$) for 5-ALA and derivatives.

Material and Methods

- **Material**

5-ALA, Bovine ceramides, cholesterol, palmitic acid and cholesteryl sulfate were purchased from Sigma Chemical Co. The derivatives n-hexyl and n-octyl ester were synthesized and characterized as described in our previous work [5]. Other reagents were HPLC grade.

- **Partition coefficients determinations**

The partition coefficients were determined with 60 μg/mL 5-ALA or ALA n- octyl ester in aqueous solution or in a liposomal system. The $K_{O/W}$ for the 5-ALA and its ester derivatives were determined in the aqueous phase after equilibrium with octanol. The $K_{S/W}$ and $K_{S/V}$ were determined in the aqueous phase after mixing 5-ALA or 5-ALA derivatives in aqueous solutions or liposomal system with approximately 300 mg excised hairless mouse skin. In both experiments, the aqueous phase before (C_1) and after partition (C_2) [6] was assayed by derivation reaction [7] followed by HPLC with fluorescence detection at λ excitation/emission= 378/464 nm**.**

- **Preparation of *stratum corneum* lipids liposomes (SCLLs)**

Liposomes with lipidic composition based on 50% ceramides, 28% cholesterol, 17% palmitic acid and 5% cholesteryl sulfate were obtained by mechanical dispersion technique followed by extrusions [8]. α-tocoferol was included to this formulations at 1 mol%, as antioxidant. These lipidic mixture (5 mg lipid/ml) was dissolved in chloroform: metanol (2:1 volume ratio) in a glass vessel. Then, the lipidic mixture was sonicated and. dried under nitrogen flux until deposition of the film on the wall of a glass vessel. After that, the aqueous phase containing drug was added (TRIS-EDTA buffer, pH 7.0) at the ratio of drug: lipids 1:3. The hydrated preparation was heated and extruded at 75ºC through polycarbonate filters of pore size 1 µm.

Results and Discussion

Figure 2 shows the partition coefficients found for 5-ALA and derivatives.

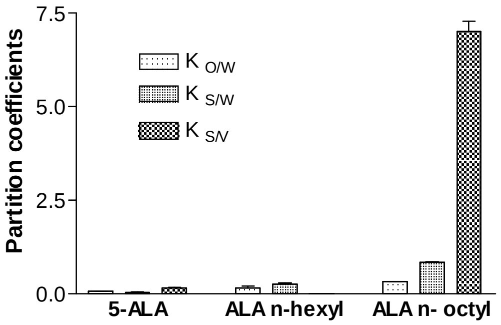

Figure 2: Partition coefficients values for 5-ALA, ALA n-hexyl ester and ALA n- octyl ester. Values are expressed as means ±SD, n=3 for each group. ($K_{S/V}$ for ALA n- hexyl ester was not determined).

Table 1: Statistic data for partition coefficients.

Partition coefficients	*Comparision*	*Significance*
$K_{O/W}$	5-ALA x ALA-hexyl	P >0.05
	5-ALA x ALA-octyl	P < 0.01 *
	ALA-hexyl x ALA-octyl	P< 0.05 **
$K_{S/W}$	5-ALA x ALA-hexyl	P< 0.01 *
	5-ALA x ALA-octyl	P<0.001 ***
	ALA-hexyl x ALA-octyl	P<0.001 ***
$K_{S/V}$	5-ALA x ALA-octyl	P=0.0009 ****

$K_{O/W}$ values for the ALA-n-hexyl ester and ALA n-octyl ester were approximately 2.6 and 5.6 times higher compared to 5-ALA, respectively. $K_{S/W}$ values to ALA-n-hexyl ester and ALA n-octyl ester were approximately 7.7 and 25.8 times higher than 5-ALA, respectively.
Finally, $K_{S/V}$ value for ALA-n-octyl ester was approximately 48.6 times higher than 5-ALA**.**

Conclusions

The results of partition coefficients have showed that the derivatives ALA n-hexyl and ALA n-octyl ester are more lipophilic than 5-ALA (higher $K_{O/W}$). The use of more lipophilic derivatives in topical PDT could raise the induction and PpIX accumulation, improving the 5-ALA biodisponibility in the tissue.
The $K_{S/W}$ and $K_{S/V}$ have confirmed the higher skin affinity of the derivatives comparing to 5-ALA, which is a desirable characteristic for drug cutaneous delivery, especially in the topical PDT. Is also desired for topical formulation a delivery system with high interaction into the skin and low systemic toxicity. The liposomal system with similar composition to *stratum corneum* (SCLLs) shows these characteristics and has improved the 5-ALA derivatives skin affinity compared to 5-ALA ($K_{S/V}$ higher than 5-ALA).

Acknowledgements

This work was supported by FAPESP.

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DD-18 DICLOFENAC/PHOSPHOLIPID INTERACTIONS IN LIPOSOMES AND MONOLAYERS

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Introduction

Sodium diclofenac (SD, figure 1) is a potent non-steroidal anti-inflammatory (NSAI) with analgesic and antipyretic effects used for the treatment of rheumatoid arthritis and osteorarthritis [1]. As with others NSAIs drugs, allergic reactions and necrosis have been reported with the drug [2]. This compound is poorly water soluble (17.8mg/L), but has a high partition coefficient (log P = 4.0) and so tends to accumulate between the polar head groups and the hydrophobic chains of the phospholipids membrane [3].

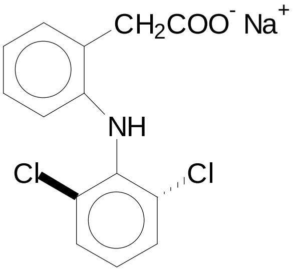

Figure 1: Chemical structure of sodium diclofenac

The monolayer model system has been applied to investigate the interfacial behavior of drug/phospholipid interactions. Experiments with monolayers have the advantage that the arrangement of molecules can be easily controlled by changing the molecular area and the surface pressure of the monolayer.

In this work, we have studied the interaction of SD with phosphatidylcholine using Langmuir monolayers and liposomes.

Experimental methods

- **Materials**

The soya phosphatidylcholine (Epikurom 200, average mol wt 750) was supplied from Lucas Meyer, Hamburg). L-α-Dipalmitoylphosphatidyl choline (DPPC) specified 99% pure (Avanti Lipids). Sodium diclofenac mol wt 318.1, (Ciba Novartis) was re-crystallized from hot ethanol. Tris (hydroxymethyl) aminomethane (Sigma Chemical Co, St Louis, USA). Organic solvents (chloroform, ethanol, and methanol) obtained from Merck S.A., Brazil, were of the best grade available. Water was ultra pure and obtained from a Milli Q Plus system (Millipore).

- **Method**

DPPC/SD Monolayer

SD was dissolved in chloroform/methanol (95:5 v/v) and mixed with chloroform solutions of DPPC. The resulting monolayers contained SD molecules embedded in a phospholipid matrix and spread on the aqueous sub phase (50µL). Several relatives concentrations of SD in the films were used varying from the 4 mol% up to 60 mol%. The surface pressure measurements were carried out by means of KSV 5000 Langmuir trough mounted in a class 10 000 clean room.

Liposomes

Multilamellar liposomes were prepared dissolving Epikuron 200 and SD (0, 0.63; 1.27, 2.5) mg/mL in chloroform and the solvent removed under vacuum. Small unilamellar liposomes were obtained by sonication in aqueous buffer solution of 10 mM Tris/HCl, pH 7.2. (Ultrasonic Liquid Processor, Heat System). The liposomes dispersions were filtered and the liposome suspensions (100 µL) spread at the constant area (35.6 cm^2) by means of a micro syringe. The sub phase used was 10 mM Tris/HCl, buffer and the temperature was 20.1°C. Variations of the surface pressure with time were recorded. Zero time was at the beginning of the spreading procedure.

Results and discussion

We observed for DPPC the presence of SD even in small concentrations produced monolayer expansion in the condensed region of the film and in smaller concentrations than 12 mol% there is condensation in the monolayer in the gaseous regions (Figure 2).

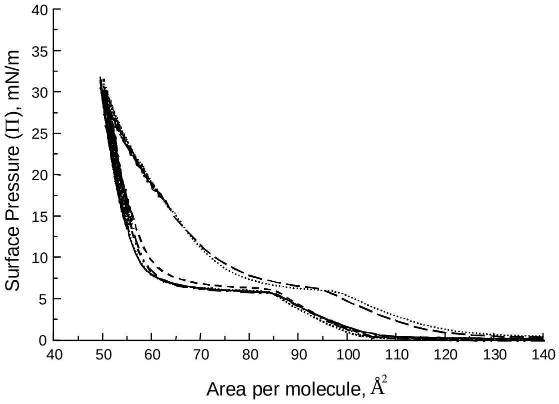

Figure 2**:** Surface Pressure vs. area per molecule isotherms for mixed DPPC/Diclofenac (T=20ºC). 0 mol% (—), 4 mol/% (-.-.-), 8 mol% (♦♦♦), 12 mol% (----), 20 mol% (-¨-¨), 40 mol% (-·-·-),60 mol% (.......).

The variation of the surface pressure versus time at constant surface area after spreading different concentrations of SD into the liposomes indicated an immediately apparent increase in the surface pressure. (Figure 3).

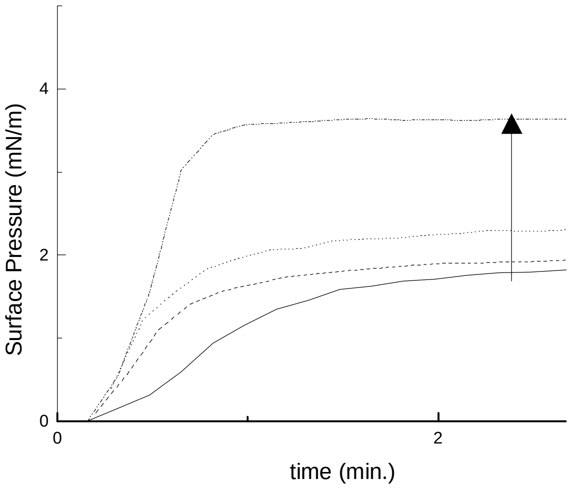

Figure 3: Variation of surface pressure with time after spreading liposome. Key: → 0, 0.6, 1.25, and 2.5 mg/mL of DS loaded liposome.

Conclusion

It was concluded that the incorporation of SD in the monolayers has a deep effect on the isotherm Π-A. This effect was directly related with the increase in the drug concentration in the liposomes and the monolayers systems.

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This work was supported by FAPESP and CNPq.

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DD-19 FIRST STEPS TO PRODUCE A GENERIC FORMULATION FOR AMPHOTERICIN B

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Introduction

Amphotericin B (AmB) remains the drug of choice for the treatment of systemic fungal infection in immunocompromised patients [1]. The main problem in using this compound consists in its high toxicity, mainly a nephrotoxicity, which causes the suspension of treatment [1]. Regardless of those important side effects, AmB has been used for more than 40 years because of its effectiveness [2]. The most common commercial form of AmB, Fungizon®, consists of a complex between AmB and sodium deoxycholate, a bile salt. The Brazilian government spent 250 thousand dollars in 1997 buying this product [3]. On the other hand, some countries have been successfully using new pharmaceutical delivery systems such as liposomes or AmB lipid complex systems, but they could be 1000 times more expensive than Fungizon®[4].

The marketing of generic products has been increasing continuously in Brazil since their approval in 1998 [5]. The production of a generic form for AmB in Brazil will permit us to reduce the price of this compound in the Brazilian market. However, the production of a micelle solution of AmB is difficult because of its physicochemical properties.

The aim of this work was to produce a generic formulation for AmB based on the formation of an AmB-sodium deoxycholate complex, and to evaluate the formation of this complex by spectroscopy.

Experimental methods

Materials

AmB, ordinary sodium deoxycholate (97%) and sodium deoxycholate monohydrate (99%), were purchased from Spectrum, USA and Sigma, USA, respectively. Sodium phosphate monobasic (monohydrated) and dibasic (anydrous), sodium hydroxide (NaOH), and hydrochloric acid (HCl) were obtained from Fisher Scientific, USA.

Preparation of AmB micellar solution

400 mg of AmB was dissolved in a 22.6mM sodium deoxycholate solution previously prepared in buffer (pH 7.4). The mixture was stirred at room temperature for one hour, cooled to 7ºC, and 1700 µl of 1.0 N NaOH solution was added under constant agitation until complete solution occurred. While the solution (pH 12.25) was vigorously stirred, 1700 µl of 1.0 N HCl was added. Stirring was continued at room temperature for two hours, after which buffer solution pH 7.4 was added to a final volume of 40 ml. Three batches were prepared using this technique. One with sodium deoxycholate monohydrate (**Batch 1**) and two with ordinary sodium deoxycholate (**Batch 2 and 3**). For all batches an AmB free micellar solution was prepared as a control.

Lyophilization of solutions

Each batch of product was lyophilized as follows. The product (3ml) was placed into a scintillation vial (with a volume capacity of 12 ml). The vials were emerged into liquid nitrogen for 20 minutes; then, they were placed on the lyophilizer for an overnight lyophilization cycle, under vacuum. After 12 hs, the vacuum was broken and the vials were hermetically closed.

Spectroscopical evaluation

Absorption spectra of the three batches were obtained by dispersion of the products in water at an AmB concentration of 5×10^{-6}M. The spectra were recorded against a blank consisting of AmB-free micelles, in the reference beam. All spectra were recorded with a Cary 118-Olis Spectrophotometer (Bogart, GA - USA), at 25ºC, using a 1 cm cuvette path-length.

Results and discussion

The incorporation method used was able to produce micelle solutions. The three batches of product presented a visual aspect and a final pH quite similar to Fungizon®. Therefore, in spite of their different degree of purification, both surfactants had the same behavior.

The spectra obtained from the **Batch 1**, **2** and **3** show the same shape as Fungizon® in water (**Figure 1**). This result permits us to conclude that the AmB-surfactant complex formed was similar. The analysis of these spectra in methanol (results not shown) reveal that all batches exhibited a variation in their AmB content, which was due to product lost during the process of manufacture. However, when the correction for concentration was made, the spectra were alike (Figure 2 and Figure 3). The lyophilizaton process did not affect the stability of AmB-surfactant complex formed. The spectra of all batches after lyophilization maintained the same format and intensity as those before lyophilization.

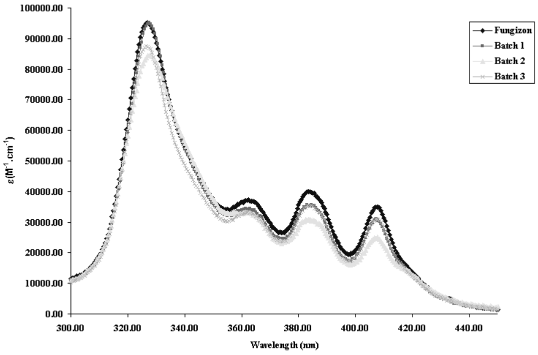

Figure 1 - Absorption spectra of products in water at 5.10^{-6}M

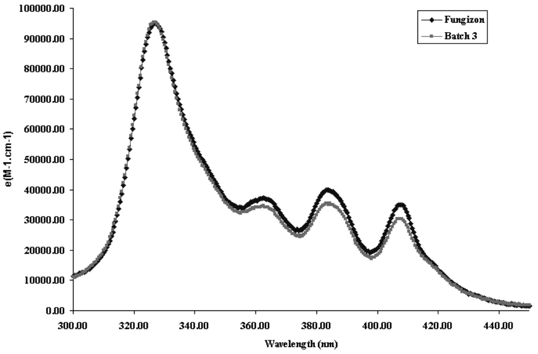

Figure 2 - Absorption spectra of products in water at 5.10^{-6}M after correction

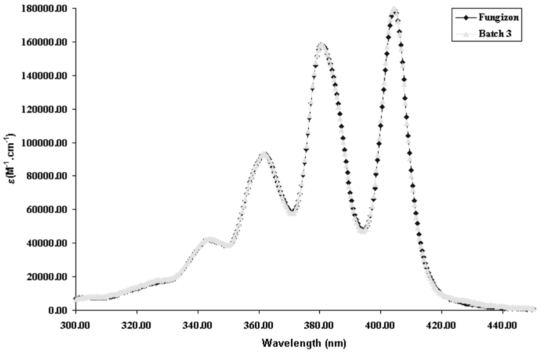

Figure 3 - Absorption spectra of products in methanol at 5.10^{-6}M after correction

Conclusion

Normally, similar intravenous pharmaceutical products are not considered as generic forms because they do not have an absorption step for being bioavailable. However, when the product formed is a complex, like Fungizon®, studies about behavior in biological fluids are very important. Only after that, is it possible to predict a similar behavior in the biodistribution process. This study has shown that the micelles produced were comparable with those existing in the Fungizon® product. This was the first step to produce a generic form for AmB. Therefore, to confirm its activity against fungal cells, in vitro and in vivo studies will be performed in the future.

Acknowledgment

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Estabelece o medicamento genérico,dispõe sobre a utilização de nomes genéricos em produtos farmacêuticos e dá outras providências.

DD-20 EFFECT OF FREE AND LIPOSOME-ENCAPSULATED HETEROPOLYSACCHARIDE FROM *ANACARDIUM OCCIDENTALE L.* AGAINST EXPERIMENTAL *S. MANSONI* INFECTION

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Introduction

Many polysaccharides have been considered as immunomodulator agents and are classified as biological response modifiers. Their biological effects, namely antitumoral and antiviral activities are said to be a consequence of this fact [1,2]. The heteropolysaccharide extracted from the *Anacardium occidentale* gum (P-JU) is composed of Gal, Ara, Glc, Rha, Xyl and Glc A in molar ratio 82:4:6:2:1:5 [3]. It has shown *in vivo* and *in vitro* its antitumoral activity against Sarcoma-180 tumors [4].
Previous studies have shown that the sulphated derivative of the $(1\rightarrow3),(1\rightarrow4)$-$\alpha$-D-glucan from the lichen *Ramalina celastri* is effective against the inflammatory process and hepatic injuries caused by *S. mansoni* infections [5].
Liposome encapsulation has been used to improve biological activity of drugs [6]. The aim of the present work describes the effects of the free and liposome-encapsulated P-JU against experimental *S. mansoni* infection.

Experimental methods

- **Materials**

S. mansoni (BH strain) was obtained from Laboratório de Malacologia, UNICAMP, SP, Brazil. Albino Swiss mice, female, 35-40 days old, 22.0 ± 2.0 g weight were obtained from LIKA/UFPE. The native polysaccharide from *A. occidentale* was extracted according to Menestrina *et al.* [3].

- **Liposomes preparation**

P-JU liposomes loaded were prepared according to Amselen *et al.* [7]. Liposomes were composed of a mixture of soya phosphatidylcholine, cholesterol and sterylamine. The thin lipid film obtained after evaporation of solvents was rehydrated with buffer phosphate solution. The suspension was sonicated to obtained liposomes with a size of 210 ± 30 nm. P-JU encapsulation rate into liposomes was determined by the phenol-sulfuric method, after the separation of

phenol-sulfuric method, after the separation of

the non-encapsulated P-JU after ultra-centrifugation.

- **Biological assay**

Six groups (n=10/group) were infected by *S. mansoni* (caudal infection, 150 cercariae/mice) and treated with a single i.p. dose of the drug 24 hours after infection, as follows: G-1, control group (150 mM NaCl); G-2, Free P-JU (100 mg.kg^{-1} /150 mM NaCl); G-3, Liposome-polysaccharide (similar to G-2); G-4, Empty liposomes. The other groups were treated 42 days after infection, under similar doses and experimental conditions, namely: G-5 and G-6, free and liposome-encapsulated P-JU, respectively.
P-JU biological activity was evaluated by the presence of *S. mansoni* eggs into faeces according to Kato-Katz method since 38th day after infection. Animals were killed at 56th day, and porta-hepatic system perfusion was then undertaken.
In vivo toxicity was evaluated by treating mice (n=10/group) with free and encapsulated P-JU, using 150 mM NaCl and empty liposomes as controls. Histopathological analysis on liver, intestine, kidneys, lungs heart and spleen was performed.

- **Statistical analysis**

Results are expressed as average ± standard deviation (M ± sd), and submitted to variance analysis and the Tukey test. Values compared at the significance level of $p \leq 0.05$ are considered to be statistically valid as follows: similar letters (*a* or *b*) mean $p > 0.05$; different letter (*a* and *b*) mean $p \leq 0.05$.

Results and discussion

Free and encapsulated P-JU were tested *in vivo*, in order to evaluate their toxicity. No toxic effect was observed.

Table 1 shows a statistically significant reduction in *S. mansoni* eggs elimination when animals were treated with liposome-encapsulated P-JU. Free P-JU reduced egg elimination in 19.6 and 58 %, for treatments undertaken at 24 hours and 42 days after infection (when worm eggs were detected in *faeces*), respectively. Liposome-encapsulated P-JU improved these performances (reduction of 82.1 and 98.8 %, for the same experimental conditions, respectively). In both cases, one can observe the efficacy in the proposed therapy.

Table 1
Effect of the treatment with free and liposome-encapsulated P-JU on *S. mansoni* egg elimination

GROUP	*S. mansoni* eggs/g (M ± sd) (days)			
	38th	42th	49th	56th
1	0	43.2 ± 20.0	390.0 ± 153.5	336.0 ± 51. 8^{a}
2	0	72.0 ± 19.6	462.0 ± 230.5	270.0 ± 98.7^{b}
3	0	30.0 ± 15.4	612.0 ± 130.7	60.0 ± 20.5^{c}
4	0	24.0 ± 17.9	522.0 ± 233.8	176.0 ± 67.1^{d}
5	0	60.0 ± 24.0	296.0 ± 12.8	141.0 ± 49.9^{d}
6	0	60.0 ± 22.0	504.0 ± 233.4	40.0 ± 19.9^{c}

Table 2 depicts the effect of the proposed treatments considering the number of adult worms recovered from porta-hepatic system perfusion at 56th day. A statistically significant reduction (54.2 %) was observed only when liposome-encapsulated P-JU was administered 42 days after infection.

Table 2
Effect of the treatment with free and liposome-encapsulated P-JU on *S. mansoni* adult worms

GROUP	Male	Female	Coupled	Total
1	29.4 ± 14.11	8.0 ± 2.9	2.8 ± 1.0	38.0 ± 16.0^{a}
2	20.0 ± 7.3	9.4 ± 3.0	6.4 ± 2.1	29.4 ± 10.1^{a}
3	23.0 ± 8.1	8.8 ± 3.7	4.0 ± 1.6	31.8 ± 14.0^{a}
4	22.8 ± 11.2	8.4 ± 3.8	2.4 ± 1.8	31.2 ± 10.3^{a}
5	14.2 ± 6.9	7.0 ± 2.9	4.2 ± 2.3	21.2 ± 10.3^{a}
6	12.0 ± 6.7	5.4 ± 2.6	3.2 ± 1.1	17.4 ± 7.1^{b}

These results were similar to those described by using a sulphated derivative of a $(1\rightarrow3),(1\rightarrow4)$-$\alpha$-D-glucan from the lichen *Ramalina celastri* (10 $\mathrm{mg.kg^{-1}}$) [5]. A reduction of egg elimination and adult worm recovery (81 and 80%, respectively), was also detected. Such an effect is possibly related to the immunomodulator activity of polysaccharide. This hypothesis is now under investigation in our lab. Moreover, the improvement of liposomes encapsulation on the anti-infections activity of P-JU was observed.

Conclusion

These results suggest that liposomes containing P-JU can be a new therapeutic possibility for treatment of *S. mansoni* infection, considering their stability and absence of *in vivo* toxicity.

Acknowledgements

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DD-21 CONSTRUCTION OF LIPOSOMES CONTAINING A POOL OF ANTIGENIC MEMBRANE PROTEINS FROM *Pasteurella multocida*

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Introduction

Pasteurella multocida is one of the most fascinating bacterial pathogens, the etiological agent of “Pasteurellosis”. It causes a wide range of diseases ranging from haemorrhagic septicaemia, an acute and fatal disease, and fowl cholera to the common secondary invasions in a range of animals. It is considered as a zoonosis, occasional infections in people derived largely from animal bites [1].
Lipopolysaccharides and proteins are the two major somatic antigens exposed on the cell surface of *P. multocida* and it is possible to use them as vaccines to control pasteurellosis [2].
The conventional vaccines using attenuated or killed whole organisms may cause mild to severe reactions. This problem has prompted the development of subunit and peptide vaccines. Unfortunately, these vaccines are often poorly immunogenic in the absence of an adjuvant [3]. To date, aluminum phosphate and aluminum hydroxide are the only immunologic adjuvants approved for use in vaccines licensed by the FDA. Recent studies have examined the use of liposomes as potential immunoadjuvants. There are several advantages of liposomes: they are biodegradable and non-toxic and can be prepared quite easily with variable compositions to obtain the most efficient antigen-presenting formulation [3]. So, we concentrated our investigation in identifying which proteins are related to the activation of the immune system of the rabbit.

Experimental methods

- ***Pasteurella multocida* membrane fraction and polyclonal antibody preparation**

Membrane protein fractions and polyclonal antibody were obtained as described by Daghastanli et al. [4].

- **Solubilization of *P. multocida* membrane proteins**

Several detergents as: 1% (w/v) SDS, 4mg/ml CHAPS, 3mg/ml CHAPSO, 5% (w/v) Polidocanol and 1% (w/v) Tween 20 were tested to solubilized antigenic membrane proteins.

- **Proteoliposomes preparation**

Proteoliposomes were formed by co-solubilization of lipids, protein and detergent using a relationship of 10:1:15 (w/w). Removal of the detergent from the mixture was done using two methods: (i) dialysis (ii) using Biobeads or Calbiosorb hydrophobic resins. The efficiency of detergent removal from the mixture was accomplished by SDS concentration. The incorporation of antigenic proteins was monitored through quantification of protein, SDS-PAGE and Western Blotting assays.

Results and discussion

A typical preparation of membrane fraction from *P. multocida*, produces about 8 mg/ml protein. The solubilization assay of membrane proteins (0.5 mg/mL) with different detergents showed that 1% (w/v) SDS was the most efficient detergent solubilizing ~85% of the proteins present in the membrane fractions using 2 h of incubation time.
The electrophoretic profile of membrane fraction and solubilized extract was practically the same (Fig. 1, lanes A and B). This result indicates a complete solubilization. After western blotting, almost all proteins showed immunogenic activity, since they were recognized by a polyclonal antibody developed against whole inactivated bacteria (Figure 1 lane D and [4]).

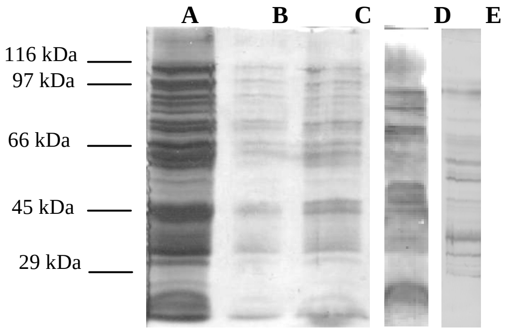

Figure 1. SDS-PAGE of bacterial membrane proteins. Silver stained proteins: (A) membrane fraction; (B) SDS-solubilized proteins (C) protein present in proteoliposome. Western blotting: (D) SDS-solubilized protein (E) protein present in proteoliposome.

The best condition for obtaining the proteoliposomes was using a relationship lipid:protein:detergent of 10:1:15 (w/w). Since the incorporation of proteins into liposomes by the co-solubilization method requires the complete removal of detergent, three methods were tested.

Table 1: Incorporation of solubilized proteins into DPPC liposomes

Detergent	Removal Method	(%) Incorporation	(%) SDS Removal
SDS	Calbiosorb	75.0	99.5
Polidocanol/SDS	Calbiosorb	67.0	99.5
SDS	Biobeads	2.9	94.0
SDS	Dialysis	10.0	55.0

The removal method of SDS affects the incorporation of proteins into liposome. Although Biobeads remove about 90% SDS, only 3% of protein was incorporated into liposome. On the other hand, dialysis removed about 55% of the SDS present and resulted in 10% reconstitution. Calbiosorb revealed the best efficiency in removing SDS (95% SDS), even in a mixture of SDS and Polidocanol. This resin leads to 75% incorporation of protein when SDS was used alone and to 67% of protein incorporation when SDS was used in association with polidocanol (Table 1).

SDS PAGE shows a wide range of proteins incorporated into liposome. These proteins showed similar profile as revealed for solubilized extracts, suggesting a randomically incorporation (Fig. 1, lane C). Among the incorporated proteins, many of them revealed immunogenicity in western blotting experiment (Fig. 1, lane E).

Conclusion

Preliminary studies showed that these proteoliposomes can induce immunity in rabbits living in a contaminated ambient

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DD-22 EFFECT OF THE ZETA POTENTIAL AND IONIC DISSOCIATION CONSTANT ON THE RATE OF DECOMPOSITION OF CEFACLOR OF SMALL UNILAMELLAR LIPOSOMES

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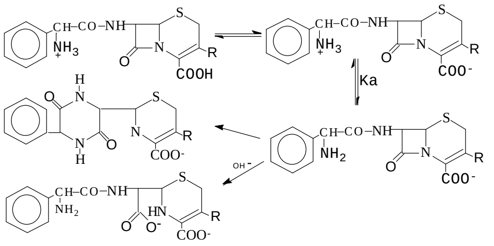
Introduction

The presence of surfactants in pharmaceutical preparations can significantly influence drug stability. Cationic unilamellar liposomes can bind ions on its surface and this association depends on the ionic dissociation constant (α) of the aggregate. The local concentration of counter-ions at interface of vesicles constitute one of the more important parameter of drug rate decomposition analysis. In this study sonicated liposomes obtained from mixtures of dimethyldioctadecylammonium (DODAC) and soya phosphatidylcholine (PC) were used to analyze the effect of ionic vesicles on the intramolecular decomposition of β-lactam cephaclor (Scheme I). The zeta pontential and the dissociation degree of mixed vesicles were determined for several molar proportions of PC and DODAC and related with the rate of decomposition of cephaclor.



Scheme I

Methods

Vesicles containing several molar proportions of PC and DODAC were prepared by sonication in Tris/HCl 0.01M, pH 7.8 buffer. The reaction were started by adding 100μl of an aqueous10^{-3}M cephaclor solution to 1.5mL of the vesicles preparation. The rate of unilamellar decomposition (k_ψ) of cephaclor was followed by spectrophotometry. The k_ψ was determined as a function of vesicle concentration.

Physical parameter such ionic dissociation constant was determined measuring the bind local concentration of chloride by ionic capture [1]. The bind chloride proportion (β) was determined according the relation between the concentration of chloride bind to DODAC and the total concentration of DODAC. Then it was calculated α=(1-β). The variation of the charge density was obtained by changing the mole ratio of lipid/DODAC in vesicle preparation.

Zeta potential was determined in a Brookhaven Instruments, Zeta Plus model, with the PALS technique. This analysis gives relatives measurements of ions mobility.

Results

The results show that the zeta potential increases significantly with the proportion of DODAC in the structural amphiphile mixture until about 70% of DODAC (figure 1).

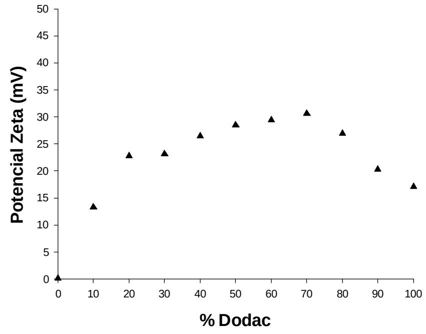


Figure 1 – Zeta potential of DODAC/PC vesicles (DODAC/PC 2.5mM in NaCl 5 mM).

The binding of Cl^- íons in the cationic vesicles surface is directly proportional to DODAC concentration in the amphiphile mixture reaching at plateau in high amphiphile concentrations (figure 2), as previously observed in the literature [2].

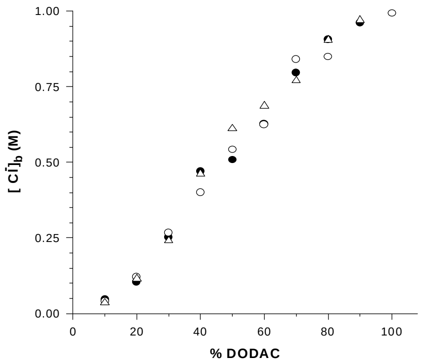


Figure 2 - Binding of Cl^- ion at DODAC/PC liposomes surface by ionic capture (NaCl 0,005 M, HCl 2 x 10^{-4} M) ● 30°C, O 36°C e Δ 45°C.

The effect of the charge on the rate of the intramolecular decomposition of cephaclor was investigated by increasing the molar fraction of cationic amphiphile (DODAC) in vesicles of PC (Figure 3). The values of k_ψ increased with total amphiphile concentration, ([DODAC] + [PC], at any DODAC/amphiphile ratio, reaching a plateau at high amphiphile concentration for all amphiphile mixtures.

The kinetic data in figure 3 was analyzed quantitatively using pseudo-phase ion-exchange model (PPIE) [3], which adequately describes the effect of a charged supramolecular aggregate on the unimolecular decomposition of an charged substrate (Scheme II).

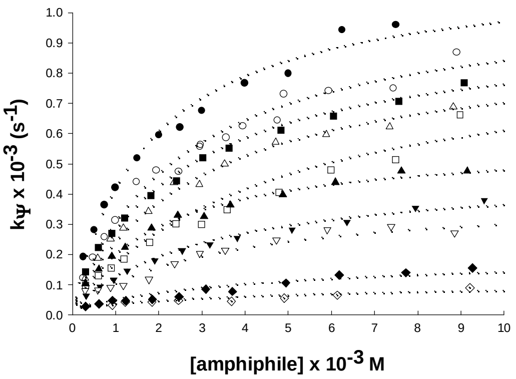
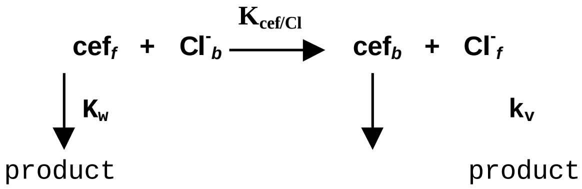


Figure 3 – Effect of liposomes of DODAC/PC (0.01M) in the constant of decomposition rate of cefaclor (buffer TRIS/HCl 0,01M, pH 7.80, μ 0,01, T=30°C, [cef] 6,25 x 10^{-5} M) • DODAC 100%, O PC 10%, + PC 20%, △ PC 30%, PC 40%, ▲ PC 50%, ▼ PC 60%, ∇ PC 70%, ♦ PC 80%, ◊ PC 90%.



Scheme II

The rate of cephaclor decomposition in charged vesicles were analyzed with equation 1, k_v and $K_{cef/Cl}$ can be fitted

$$\boxed{k_\psi = \frac{k_w + k_V.K_{Cef/Cl}.(Cl_b/Cl_f)}{\left(1+K_{Cef/Cl}.(Cl_b/Cl_f)\right)}} \qquad (1)$$

The maximum rate acceleration of intramolecular decomposition of cephaclor produced by DODAC vesicles was about 45 fold, calculated by comparing the maximum observed rate constant $k_{\psi max}$ with the rate constant in water k_w at 30°C. The value of k_v increased with the mole percent of DODAC, reaching a k_v/k_w ratio of about 56 fold [k_w=2,117x10^{-5} (s^{-1})] [4].

Conclusion

Small unilamelar liposomes accelerate the intramolecular decomposition of the antibiotic β-lactam cefaclor. The binding of cephaclor on ionic surface is dependent on charge density of vesicles. The values of k_ψ and K_v increase with concentration of íons Cl^- on the cationic vesicles surface. The data can be analyzed quantitatively using pseudo-phase ion-exchange model (PPIE). The relation between k_ψ and k_w reacts lineally with the proportion of DODAC in the vesicles.

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DD-23 PREPARATION AND CARACTERIZATION OF MICROCAPSULES FOR CONTROLLED RELEASE OF DICLOFENAC

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Introduction

Dosage forms of controlled release for oral administration have been recognized as a significant progress in the therapeutics, where a prolonged and more uniform concentration of drug is found, providing several advantages comparing to conventional dosage forms. Among of the controlled drug release system, the microcapsules are considered efficient [1].

Microcapsules allow the encapsulation of microscopic amounts of drug or substances for diagnosis purpose, with a thin polymeric film. It is appropriate for oral or parenteral administration of drugs.

Ethylcellulose is probably the most widely used water-insoluble polymer in film coating due to its good film-forming property [2]. It allows good morphology microcapsules obtaining through the emulsification/ solvent evaporation method, using the water as non-solvent [3, 4, 5].

Among the microencapsulated drugs described in the literature, we found the antiinflamatory classes due to the accentuated side effects they caused.

The present study examines the ethylcellulose microcapsules obtaining and controlled release of microencapsulated sodium diclofenac.

Experimental Methods

Materials

Ethylcellulose (Sigma) and sodium diclofenac (Galena). All other chemicals were of analytical grade.

Preparation of ethylcellulose microcapsules by emulsification/solvent evaporation method

The ethylcellulose microcapsules were prepared for the emulsification/solvent evaporation method, using the water as non-solvent. The ethylcellulose solution in ethyl acetate containing the drug suspended was dropped into the surfactant aqueous solution, Tween 80, under stirring. After the dropping, the solvent evaporation was accomplished in rotary evaporator. The microcapsules were isolated through centrifugation, washed twice with water and then dried.

Caracterization of microcapsules

The morphology and the particle size distribution of obtained microcapsules were analyzed by optical and scanning electron microscopy (Jeol T330A).

Determination of sodium diclofenac content

The sodium diclofenac content was determined by extraction in ethanol under stirring for one hour. After extraction, the sample was filtered and analyzed in an UV/VIS spectrophotometer at 277nm.

Infrared analysis of the microcapsules

Samples of ethylcellulose microcapsules without and with drug were submitted to the infrared analysis for verification of drug encapsulation. The samples were prepared in KBr disks.

In vitro release tests

The drug release tests were carried out into simulated gastric and enteric environment, both without enzymes.

Results and Discussion

The methodology used for preparation of the microcapsules involving emulsification/ solvent evaporation method allowed the good morphology micropaticles obtaining with spherical shape, flat surface and diameter less than 50µm.

In the study for the development of inert microcapsules, the concentration of ethylcellulose solution and ratio of organic phase (ethyl acetate) about the morphology and yield of microcapsules were observed, showing that the appropriate concentration of the ethylcellulose is 0.1%, because it allows the microparticles obtaining with low proportion of amorphous agglomerates and film formation. Ratio of aqueous phase (0.8% Tween 80 solution) and organic phase (ethylcellulose solution in ethyl acetate) was of 3:1.

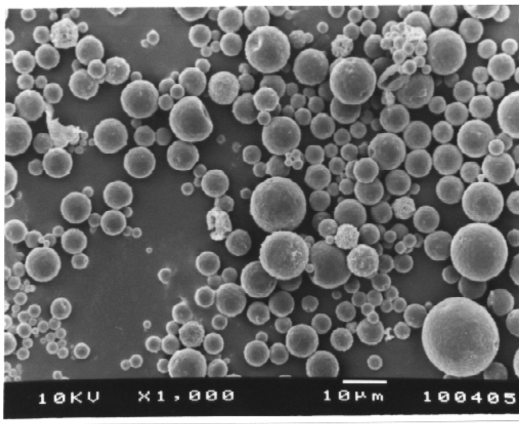

Figure 1. Photomicrography of ethylcellulose microspheres.

Through the *in vitro* drug release tests a controlled drug release profile was observed, what proves the effectiveness of the ethylcellulose microcapsules as controlled drug release systems.

Conclusion

The ethylcellulose, for being a polymer with high capacity of film formation, allows the obtaining of spherical, flat and with appropriate size distribution microparticles, through the emulsification/ solvent evaporation method. The obtained microparticles provided the encapsulation and controlled release of sodium diclofenac.

Acknowledgements

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DD-24 SOME FACTORS AFFECTING THE PREPARATION OF PROPOLIS MICROCAPSULES

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Introduction

Microcapsules are minute containers ranging in size from 1 to 1000 µm. These systems have considerable attention in recent years, because of their potential use as drug delivery system. Microencapsulation can improve the stability, release and absorption time, mask odor and taste of the encapsulated drug [1,3,4].

Propolis is a balsamic resin, with complex composition, viscous consistence and dark brown color, collected by bees from plant exudates (resinous, rubbery and balsamic substances) to reinforce honey combs, entrance walls and cover cracks in the hive. It has wide application in medicine, cosmetics and food industry due to its versatile biological activities. These include antimicrobial, fungicidal, antiviral, antiulcer, immunostimulating, hypotensive and cytostatic activities. Propolis has an unpleasant bitter taste [2,6]. Therefore, there is a need to develop a propolis preparation that masked its unpleasant taste.

The objective of this work is to evaluate some factors affecting the preparation of propolis microcapsules.

Experimental methods

- **Materials**

The following chemicals were used: polyvinyl alcohol (PVA), beeswax, gelatin, sorbitol, manitol, dextrose, propolis and alcohol (96°GL).

- **Propolis extract preparation**

Propolis extract (10%, w/w) was prepared with alcohol (96°GL) using turbo extraction [5]. Relative density, pH and dryness residue has been determined.

- **Preparation technique evaluation**

Propolis extract was dispersed in aqueous fraction using two techniques: direct mixture (n=3) and drip (n=3), with magnetic agitation by 30 minutes. The samples were lyophilized by vacuum drying and were re-dispersed in an adequate volume of distilled water for analysis. All steps were investigated by using optical microscopy.

- **Microencapsulation using cryoscopic and osmotic agent**

Microcapsules suspensions were prepared, with and without dextrose (5%, w/w), using manitol (1, 2.5, 5, 10 and 20%, w/w) or sorbitol (1, 2.5, 5, 10 and 20%, w/w). The samples were lyophilized by vacuum drying and were re-dispersed in an adequate volume of distilled water for analysis. All steps were investigated by using optical microscopy.

- **Microencapsulation using wall reinforcement**

-Beeswax
Aqueous beeswax (5%, w/w) dispersion was prepared by heating. Under magnetic agitation (30 minutes), propolis extract was dropped in beeswax dispersion.

-Gelatin
Aqueous gelatin (5%, w/w) was prepared. Under magnetic agitation (30 minutes), propolis extract was dropped in gelatin dispersion.

-Polyvinyl alcohol (PVA)
Aqueous PVA dispersion (0.5%, w/w) was prepared. Under magnetic agitation (30 minutes), propolis extract was dropped into PVA dispersion.

All samples were lyophilized by vacuum drying and were re-dispersed in an adequate volume of distilled water for analysis. All steps were investigated by using optical microscopy.

Results and discussion

Propolis extract analysis yield the propolis extract amounts indicated below (Table 1).

Table 1. Propolis extract analysis.

Test	Result	n	s	CV(%)
Relative density (g/mL)	0.8208	3	0.0008	0.0975
Dryness residue % (P/P)	4.25	3	0.0100	0.2353
pH	6.0	3	0.0005	0.0083

Preparation technique evaluation test has shown that drip technique produced better shaped microcapsules and higher quantity than direct mixture technique (DM) (Table 2).

Table 2. Preparation technique evaluation results.

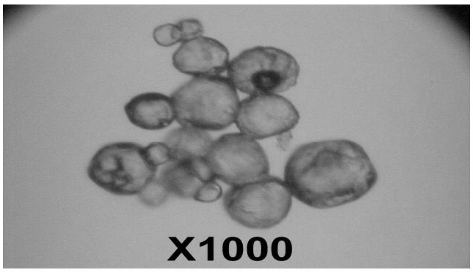
Technique	n	Results
Drip	3	Medium to small size, Spherical shape, high amount of microcapsules.
DM	3	Irregular size and shape. Few microcapsules

Microencapsulation using cryoscopic and osmotic agent test has shown that manitol (5%,w/w) is an effective cryoscopic and an osmotic protector.

Wall reinforcement tests are illustrated on table 3 and picture below.

Table 3. Wall reinforcement tests results.

Agent	Results
Beeswax	Medium to small size, Spherical shape and a few amount of microcapsules.
Gelatin	Medium to small size, spherical shape and high number of microcapsules.
PVA	Medium to big size, spherical shape and high number of microcapsules.



Picture 1– Microphotographs of propolis microcapsules with PVA.

Conclusion

This study has shown that the drip technique, to prepare propolis microcapsules, is better than direct mixture technique. Manitol (5%, w/w) has shown to be the best cryoscopic and osmotic agent. Wall reinforcement tests suggest that gelatin and PVA can be used to reinforce wall propolis microcapsules. PVA has shown the better results. Further experiments are required so as to investigate propolis microcapsules properties and to improve propolis microcapsules characteristics.

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DD-25 O/W MICROEMULSIONS FOR ORAL ADMINISTRATION OF ACTIVE LIPOSOLUBLE COMPOUNDS

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Introduction

Microemulsions are fluid, transparent, thermodynamically stable oil and water systems, stabilized by a surfactant usually in conjunction with a cosurfactant, which may be a short chain alcohol, amine or other weakly amphiphilic molecule [1-3].

The dispersed phase is made up by tiny drops with a diameter ranging from 100 to 1000 Aº [1]. They are classified as o/w, w/o and bicontinual, both phases are placed randomly in the latter [2,4].

From a pharmacotechnical viewpoint, their most important characteristics are:

- Transparency: the internal phase diameter is shorter than light wavelength.
- Low viscosity: similar to that of their organic phase; which makes behavior over time easier to be controlled.
- Thermodynamically stable: their formation is spontaneous and needs no external energy contribution.
- Isotropy: the medium where light is propagated has the same optical properties in all directions; this property ensures an homogeneous distribution of the active compound throughout the excipient.
- Diameters of drops are shorter than 1000 Aº, which makes the sterilizing filtration possible.
- Capacity to solubilize both hydrophilic and lipophilic compounds and to carry compounds having both behaviors in the same formula [3,4].
- Capacity of increase the bioavailability of some drugs, improve the prediction of some pharmacokinetic parameters, for instance, absorption and plasma levels, and reduction or elimination of administration problems with erratic absorption [5].
- Possibility to incorporate large amounts of oil, thus making it possible their use as carriers of water-insoluble drugs; they have also been successfully tested as vehicles for polypeptidic drugs [6].

A prerequisite for the pharmaceutical use is the low toxicity of their formula.

The objective of this work was to obtain a microemulsive composition for oral administration of drugs having a low solubility in water, which shows appropriate stability characteristics with a minimum toxicity and which may be elaborated through a simple method based on a computing system including all the stages of a Galenic development.

Experimental methods

- **Methodology**

A water / isopropyl myristate / polysorbate 80 / sorbitol system with a tensioactive / cotensioactive relationship = 1:2 and a work temperature of 55 ºC was selected.

1. One hundred and fifty formulations representing all the areas of the pseudoternary phase diagram were prepared. The following work methodology was adopted to represent them:

1.1 The cotensioactive was solubilized in water, the tensioactive was dispersed in the oily phase; they were submitted to a temperature of 55 ºC, mixed and stirred for ten minutes with a magnetic agitator, and placed into a stove at 37 ºC for 24 hours.

1.2 The preparations were characterized considering number of phases, aspect and capacity to solubilize dyes.

2. From the sixteen microemulsions obtained, pre-concentrated compounds were formulated with isopropyl myristate, polysorbate 80 and sorbitol in a 70% solution with the purpose of working at room temperature.

3. A stability survey was carried out in order to compare both work procedures through their behavior with monovalent, divalent and trivalent electrolytes, 3500 rpm centrifugation resistance time, and cycling between 4 ºC and 37 ºC, in addition to normal aging.

4. Identical systems were elaborated following the two procedures described above, but with the addition of 200 mg / 5 ml of alpha-tocopherol.

5 The stability of the latter was studied against the former on the basis of the same parameters.

6 Higher stability and lower toxicity systems were selected. They were characterized by their viscosity, density, internal phase size and pH.

7 Progesterone in a concentration of 20 mg / 5 ml was carried as the active compound; its stability in the formulation was studied following the B.P. assay.

Results and discussion

The results of this study are detailed in Table I and Table II.

Table I
Summary of stability data at 55 °C

System (number)	w/o/s-s (% w/w)	Normal Aging (days)	Cycling (days)	C.R.T. (min)
1	50:5:45	60	27	360
2	52:5:43	45	12	300
3	57:5:38	13	5	60
4	50:7:43	Metastable		
5	48:7:45			
6	47:8:45			

Table II
Summary of stability data at 25 °C

System (number)	w/o/s-s (% w/w)	Normal Aging (days)	Cycling (days)	C.R.T. (min)	PG (mg / 5ml)
1	50:5:45	60	26	360	12.33
2	52:5:43	60	16	90	16.45
3	57:5:38	60	30	360	6.98
4	50:7:43	60	12	150	16.15
5	48:7:45	60	14	90	18.08
6	47:8:45	60	14	120	19.97

References:
w/o/s-s: water/oil/surfactant-cosurfactant
C.R.T.: Centrifugation resistance time
PG : Progesterone

It should be noted that the systems shown in Table II did not become unstable after the addition of the active compound. The systems 1, 3, 5 and 6 were capable to keep their stability for a period longer than a month. System 1 containing 200 mg / 5 ml de alpha-tocopherol also presented acceptable parameters of stability.

Conclusion

Comparisons of the two work procedures revealed that microemulsions obtained at 25 °C were the most stable systems.

These o/w microemulsions have pharmaceutically acceptable characteristics for oral administration and keep their stability when progesterone is incorporated.

The drug solubility would be increased by addition of alpha-tocopherol to the formulations.

Acknowledgements

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DD-26 EFFECT OF SURFACTANT COMPOSITION ON THE FORMATION OF NEUTRAL LIPID MICROEMULSIONS AND INCOPORATION OF SODIUM DICLOFENAC

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Introduction

Microemulsions (ME) are isotropic, transparent, thermodynamically stable systems usually obtained from a four-component mixture containing surfactant, co-surfactant, oil phase and water [1,4,5]. Microemulsions stay stable due to free energy from the system to be zero. These systems have been investigated as drug delivery system and the advantages offered by ME include the enhancement of drug solubility and absorption and protection against enzymatic degradation [2,5,6]. A great reports number in the literature suggest the use of lipid ME for the improvement of oral absorption of drugs [2,3]. In fact, microemulsions have very promising applications in the pharmaceutical field due its ability to increase intestinal absorption of drugs, target possibility and reduction of side effects [1,3]. Since ME are formed spontaneously when exposed to aqueous media, not request high technology, temperature enhancement, or the use of organic solvents for obtaining [6]. Since the domain of the existence of ME is due to the thermodynamic behavior exhibited by the different combinations among the components, the phase diagrams can be used for that description [4,5]. The purpose of this work was to study the effect of several proportions of the components of surfactant mixture (Cremophor RH 40 / Epikuron 200) on domain of ME in the pseudo-ternary phase diagram and also to determine the physical characteristics of the system. The parameters of the incorporation of sodium diclofenac in A/O ME containing Captex 200 as oil phase was also studied.

Experimental methods

- **Materials**

Polyoxyethylenglycerol trihydroxystearat 40 (Cremophor RH 40), Basf S/A, Brasil; Propylene glycol diesters of caprylic/capric acids (Captex 200), Abitec Corporation, Janesville, USA; Soya phosphatidylcholine (Epikuron 200), Lucas Meyer, Hamburg, Germany; Chloroform, Merk, Darmstadt, F.R.Germany; Tris (hidroxymetil) aminometano (Merk, Darmstadt, F.R.Germany).

- **Methods**

Microemulsions preparation

Adequate amounts of nonionic surfactant (Cremophor RH 40) were mixed with soya phosphatidylcholine (Epikuron 200) at several proportions 1:9 to 9:1. The oil phase (Captex 200® and aqueous buffer were then added. This addition sequence enabled the dissolution of components, with slowly agitation. The mixtures were gentle mixed by 10 minutes in a temperature controlled room at 25± 0.1°C.

Physical chemical characterization

Physical chemical measurements were determined using the microemulsion A, containing 37% of surfactant mixture, 46% of phase oil and 17% of aqueous buffer and B, containing 37% of surfactant mixture, 58% of oil phase and 5% of aqueous buffer. The refractive index of ME was obtained using Abbe refractometer, Carl Zeiss-Jena. Deionized water and α-bromonaftalin were used to calibrate the instrument. The measurement of the viscosity was made using a RotoVisco Star-LP, Select instrument, calibrate with glycerin. The density was obtained using a pycnometer method. All the measures were made in a temperature controlled room at 25± 0.1°C.

Drug Incorporation

To analyze the drug incorporation the standard curve of sodium diclofenac aqueous buffer was established. Diclofenac incorporated in ME was determined by UV-Vis spectrophotometry at 277nm in function of the percent of aqueous phase and the solubility diagrams were obtained. The values of the incorporation constants (Ks) were calculated from angular coefficients of the straight line of the solubility diagrams.

Results and discussion

The obtained results show that is possible to produce clear systems like W/O and O/W liquid and gel ME using only Cremophor as a surfactant in a wide range of components combination. The addition of phosphatidylcholine to the surfactant mixture enhanced the gel domain in phase diagram. When the phosphatidylcholine proportion was increased in the surfactant mixture there was a progressive decrease of the O/A ME domain and increase the A/O domain. ME stabilized with a single soya phosphatidylcholine surfactant provide the obtaining of oil ME with small amount of aqueous phase. The density of ME varied in the range of 0.96 to 0.99 g/mL. The refractive index average values were 1.44. At water fraction volume constant, the viscosity decreases with the proportion of phospholipid in the composition of surfactant. The rheogram of system show that the viscosity increases with stress time. The solubility diagram shows that the concentration of sodium diclofenac dissolved increases with the internal phase volume of ME. The association constant obtained enhanced with the proportion of Cremophor in the surfactant composition. This phenomenon can be explained since the drug dissolution depends on the dielectric constant of ME and the hydrophilic surfactant provides this condition.

Conclusion

The results obtained showed that is possible to obtain clear and stable, liquid and gel phase ME in a wide range domain on the phase diagram. It is possible to incorporate hydrophilic drugs like sodium diclofenac in the system. The ME studied can be used as an efficient drug delivery system and also to increase the solubility of hydrophilic drugs in lipid media.

Acknowledgements

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DD-27 INCORPORATION AND RELEASE OF SODIUM DICLOFENAC FROM WATER-IN-OIL MICROEMULSION BASED ON LECITHIN

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Introduction

In the last years a lot of attention has been given to the design of new dosage forms to increase the effectiveness of drugs. This system can be used in the pharmaceutical field not only to improve therapeutic effects of drugs but also to allow decreasing in side effects.

In this way, microemulsions (ME) have been studied as drug delivery system, mainly the lipid-based systems[1,2]. The main advantage of the ME is the thermodynamic stability that makes it better than common emulsions. The obtaining of ME is a spontaneous process, providing a wide shelf life for the system[3,4]. In this work we have studied the influence of different compositions of surfactant composed by hydrogenated soya phosphatidylcoline (PC) and polyoxyethylenglyceroltrihydroxystearat 40 (Cremophor RH 40) in the ratios of 0:10 to 10:0, in order to obtain ME containing caprilic/capric acids mono/diglyceride (Capmul MCM) as oil phase (O) and buffer system as aqueous phase (W). The phase diagrams of ME were established and the physical chemistry characteristics (viscosity, density, conductivity, refractive index and droplets diameter of internal phase) of the ME as determined. The drug release using sodium diclofenac as hydrophilic model drug incorporated in water in oil ME was analyzed.

Experimental methods

- **Materials**

Chloroform (Merck, Germany); Polyoxyethylene glyceroltrihydroxystearat 40 - Cremophor RH 40 (Basf S/A Brasil); Propylene glycol diesters of caprylic/capric acids - Captex 200 (Abitec Corporation, Janesville, USA); Hydrogenated Soya phosphatidylcholine - Epikuron 200 (Lucas Meyer, Hamburg, Germany); Tris (hidroxymetil) aminometane (Merk, Darmstadt, F.R.Germany).

- **Microemulsion Preparation**

Adequate weights of nonionic surfactant (Cremophor RH 40) were mixed with hydrogenated phosphatidylcholine (Epikuron 200S) at several proportions. The oil phase (Capmul) and aqueous buffer were then added. This addition sequence enabled the dissolution of components, with slowly agitation. The mixtures were then carefully stirred for 10 minutes in a temperature controlled room at 25± 0.1°C. The regions of clear ME, sub-microemulsion (SM), gel phase (G) and separated phase were defined by pseudo ternary phase diagram.

- **Physical Characterization**

The regions defined in the phase diagram as clear ME systems, was selected for study with four different formulas containing: 7% W, 7% surfactant (PC/CR) and 86% O; 11%W, 7% surfactant (PC/CR) and 82% O; 15% W, 7% surfactant (PC/CR) and 78% O; 7% W, 7% surfactant (PC/CR) and 73%. For all formulas, the ratio of PC/CR was modified as previously described. The refractive index of ME was determined using Abbe refratometer, Carl Zeiss-Jena. The measurements of the viscosity, was made using a Brokfield-Model DV-II Programmable Rheometer. The conductivity was determined using a Digmed mod. Cd-21 conductivity meter. The droplets diameter was determined using light scattering method. To measures the density, was used picnometer method. All measurements were made in controlled temperature (25^{o}C).

- **Drug incorporation and releasing**

For the analyses of drug incorporation the standard curve of sodium diclofenac in buffer system was established. The sodium diclofenac incorporated in ME was determined by UV spectrophotometry at 277nm. The solubility diagrams were obtained in function of the percent of aqueous phase of ME. The values of the incorporation constants (Ks) were calculated from angular coefficients of the straight line of the solubility diagrams. The release of sodium diclofenac from ME was made by monitoring absorbance values in function of time, using a volume-reduced method directly in a spectrophotometer cuvette.

Results and discussion

The results obtained showed that ME, SM and gel phase were defined with success in the different regions of phase diagram. It was observed, a decrease of ME and an increase of SM domain areas, by the variation of PC/CR range in the way of 0:10 to 10:0. The W/O ME domain was observed in the ratios 0:10 to 6:4 and for O/W ME only in 0:10.

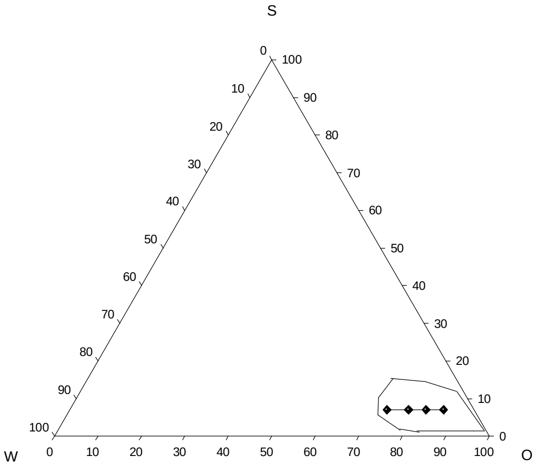

Figure 1. Phase diagram ME region studied. The straight line represents the aqueous phase proportion used. (7, 11, 15 e 20%).

It can be explained, because PC has lipophilic characteristic and decreases the incorporation of aqueous phase in the system while CR, having hydrophilic characteristic, increases the aqueous phase incorporation.

The density varied from 1.004 to 1.014g/ml, the viscosity varied from 65 to 78cP and showed a Newtonian rheological behavior. Conductivity varied in the range 26.7 to 96.7 µS/cm.

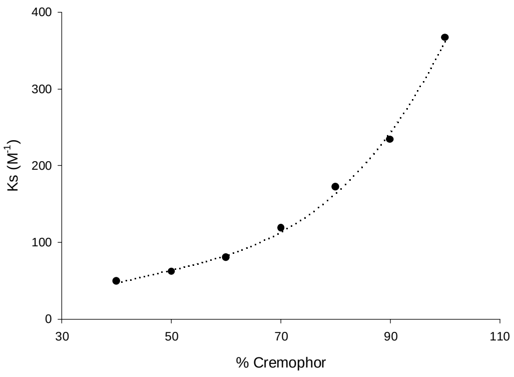

Figure 2. Variation of incorporation constant (Ks) of sodium diclofenac against ME surfactant composition.

The solubility of sodium diclofenac was directly proportional to the increase of the fraction of internal phase volume of the ME. Since the drug has a hydrophilic character, the obtained Ks´s values increase with the increase of Cremophor fraction in the surfactant composition.

The diclofenac release showed to be faster with the increase of hydrophilic component (Cremophor) in the formula, as well as the increase of aqueous phase.

Conclusion

The results demonstrated that was possible to obtain stable, optically transparent ME, using PC, CR and PC/CR as surfactant system, Capmul MCM as oil phase and aqueous buffer system as water phase. The physical characterization of W/O ME, were determined with success. An increase of hydrophilic component as well an increase of the percent of aqueous phase in the formula facilitated the release of sodium diclofenac from the water-in-oil microemulsion.

Acknowledgements

The authors wish to tanks to Fundação de Amparo à Pesquisa do Estado de São Paulo FAPESP and CNPq for financial support.

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DD-28 CHARACTERIZATION OF THE COMPLEXES FORMED BETWEEN CATIONIC EMULSIONS AND OLIGONUCLEOTIDES: INFLUENCE OF THE PEG-LIPIDS

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Introduction

Antisense oligonucleotides (ON) have been used to selectively modulate the expression of genes and to inhibit the synthesis of cellular proteins. However, ON-based therapy is compromised by the rapid degradation of ON in biological fluids and by their inability to efficiently cross cell membranes due to their both hydrophilic character and large molecular structure [1].

Lipids and especially cationic lipid based systems have gained an increasing interest due to their ability to allow efficient ON transfection. The most successful method for ON delivery is the development of cationic liposomes composed of cationic lipids and a helper phospholipid such as DOPE [2-3]. *In vitro*, these ON/liposomes complexes were shown to be delivered into the cytoplasm at an early stage of the endocytotic pathway with a marked increase of antisense activity [2]. However, i*n vivo*, the large size and the excess of positive charge result in an ON lung accumulation and a rapid uptake by tissues of the mononuclear phagocytic system [3,4].

In this context, we have recently reported triglycerides based cationic emulsions as a delivery system for ON [5]. The use of PEG-based lipids has been proposed as a strategy to mask the net positive charge and to reduce the opsonisation of the oil droplets [6]. The aim of the present work was to characterize the complexes formed between ON and cationic emulsions by size, ζ-potential determinations, fluorescence resonance energy transfer (FRET) as well as ON release rate.

Experimental methods

- **Emulsion preparation**

A typical formulation (%, w/w) consisted of medium chain triglycerides (MCT) 7.5, phosphatidylcholine (Lipoid E-80®) 2.0, α-tocopherol 0.02, poloxamer 1.68, glycerol 2.25 (PC), stearylamine 0.5 (PC/SA) and Miliq® water up to 100. For PEG-emulsions (PC/SA/PEG), 2.5 mol% of lipoid E-80 was replaced by DSPE-PEG$_{2000}$. pdT$_{16}$ (12.5 µM) was associated at the end of the manufacturing process as previously described [5,6].

- **Emulsion characterization**

The droplet size of the emulsions was estimated by laser light scattering with an N4 Coulter Nanosizer® (Hialeah, U.S.A.). The zeta potential was measured with a Malvern Zetasizer® (Malvern, U.K.).

To investigate the localization of the ON associated with the emulsions, FRET studies have been performed using NBD-PE probe and pdT$_{16}$ labeled at the 3'end with rhodamine (pdT$_{16}$–rho). Prior to the emulsion preparation, NBD-PE was incorporated into the oil phase. pdT$_{16}$–rho was added to the final emulsions at the same fluorophore molar ratio [7]. FRET was evaluated by the fluorescence intensity ratio at 520 versus 575 nm for each emulsion containing the labeled ON.

Release studies were performed in PBS pH 7.4 at 37°C. Emulsions were diluted (25-fold) in the release medium and after 5, 15, 30, 60, 120, 240 and 360 min., the medium was filtered off through a porous membrane (0.1 cut off) and the amount of free ^{33}P-pdT$_{16}$ was determined in the ultrafiltrate by liquid scintillation.

Results and discussion

The amount of pdT$_{16}$ associated with the cationic emulsions was close to 100% up to 125 µM ON since no ^{33}P-pdT$_{16}$ radioactivity was found in the external aqueous phase after the separation procedure. For PC emulsion, the association efficiency was lower, around 80%. As expected, because of its positive charge, stearylamine (SA) allowed to engage electrostatic interactions between pdT$_{16}$ and the oil droplets which have in turn favored pdT$_{16}$ association.

FRET experiments were performed to investigate the localization of the ON at the interface of emulsion droplets (Table 1). The results clearly evidenced that energy transfer between the two probes (pdT$_{16}$-rho and NBD-PE) could only occur with cationic emulsions and not with the single PC-formulation. The presence of a positive charge on the droplet surface clearly contributed to bring the polyanionic ON molecules closer to the interface. Thus, ON /SA ion-pairs should localize inbetween the interfacial film. The addition of 2.5 mol% DSPE-PEG in the composition of the film led however to the absence of transfer because of the steric hindrance induced by the PEG chains, reducing ON/SA interactions.

Table 1. Fluorescence intensity ratio (I_{520}/I_{575}) from different submicron emulsions containing NBD-PE as a fluorescent probe.

	I_{520}/I_{575}	
	Theoretical ratio in absence of energy transfer	Experimental ratio
PC	0.30	0.32
PC/SA	0.90	0.53
PC/SA/PEG	0.29	0.29

([a])IF ratio obtained at t=3h are presented, ([b])theoretical ratio was calculated from the spectrum obtained after addition of the fluorescence intensity of an emulsion sample on the one hand, and the ON sample on the other hand, separately, prepared in the same condition as the mixed emulsion/ON.

Table 2 shows ζ-potential of submicron emulsions before and after ON association. When increasing concentrations of pdT$_{16}$ were incubated with the PC/SA, ζ-potential remained unchanged and no significant changes in the size (data not shown) were detected. ζ-potential decreased, however, from +50 to + 26mV in the case of PC/SA/PEG.

Table 2. ζ-potential of submicron emulsions.

	ζ-potential (mV)		
ON (µM)	PC	PC/SA	PC/SA/PEG
0	-28±6	+49±6	+50±6
1.25	-28±6	+46±6	+48±5
12.5	-29±5	+47±6	+47±6
75	-32±4	+45±6	+38±6
125	-31±3	+44±6	+26±6

We previously (5) explained that SA was in excess in PC/SA emulsions and that the oily core behaved as a SA reservoir, replacing at the interface the SA molecules engaged in an ion-pair with ON so that ζ-potential remained constant despite association efficiencies close to 100%. Thus, the decrease in ζ-potential observed with PC/SA/PEG is consistent with the fact that the anionic charges brought by ON adsorption were not completely neutralized by SA through ion pairs, but on the contrary remained partially free in the vicinity of the PEG-chains. In these conditions, it is quite understandable that ON molecules being in a hydrophilic environment rather than in a lipidic one (PC/SA interface) were more easily released in PBS (~20 to 100% after 240 min of incubation, for example) when DSPE-PEG was used as an interfacial lipid (Figure 1).

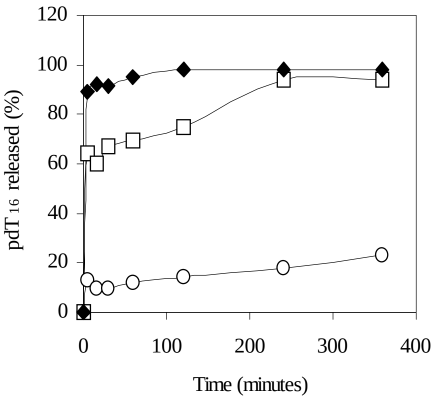

Figure 1. ON released from emulsions in PBS at 37°C. Key: (◆)PC, (○) PC/SA, (☐) PC/SA/PEG.

Conclusion

The incorporation of PEG-lipids in the cationic emulsions modifies the localization of ON at the interface, leading to an increase in the release rate.

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DD-29 STABILITY STUDY OF AMPHOTERICIN B MICROEMULSIONS

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Introduction

Interest in the Amphotericin B (AmB) has grown dramatically because of a rise in the incidence of systemic mycoses [1]. Although discovered more than fifty years ago, this compound remains the drug of choice for the treatment of life-threatening fungal infections [2]. Nevertheless, its commercially available form, Fungizon™, in which the drug is solubilized by sodium deoxycholate, can present severe acute and chronic toxicity. In order to reduce its toxicity, several studies have suggested including AmB, which is lipophilic, in lipid carriers, such as small unilamellar liposomes (Ambisome™), colloidal dispersion (ABCD, Amphocil™, or Amphotec™), and lipid complex (ABLC or Abelcet™) [3]. All of these lipid-based AmB preparations are reported to have better therapeutic indices than Fungizon™, but their use is limited by their high cost and difficult preparation [4].

On the other hand, microemulsions have been proposed as potential drug carrier systems for oral, topical, and parenteral administration [5]. These systems are easy to manufacture and are thermodynamically stable; they also offer the advantage of improved drug solubilization [6]. Recently, we have shown the potential use of microemulsion as an AmB carrier [7]. In fact, the low water solubility of this drug as well as its high rate of hydrolysis in aqueous media [8] makes the microemulsion an appealing system to stabilize AmB in a liquid medium.

The aim of this work was to evaluate the stability of AmB into two different microemulsion systems.

Experimental methods

- **Materials**

Mygliol 812™ was kindly supplied by CONDEA Vista Company, USA. Saponified coconut oil (SCO) was extracted by the Department of Chemistry of the UFRN; Sodium deoxycholate (DEO) and 1-butanol was purchased from Sigma, USA.

- **Preparation of the microemulsions**

The microemulsions systems (ME) with DEO and SCO were obtained by mixing the four components according to the **Table 1**.

Table 1 - Microemulsion formula

Component	ME-DEO [% $_{(w/w)}$]	ME-SCO [% $_{(w/w)}$]
Mygliol 812™	3	3
SCO	-	7.9
DEO	11	-
1-butanol	44	31.6
Water	42	54.5

The ideal amount of each component was previously determined by a ternary diagram study [9]. The ME-DEO and ME-SCO containing AmB were obtained by dissolving the drug, 2×10^{-3}M and 1×10^{-3}M, respectively, directly into the ME. For each preparation one ME was prepared without AmB.

- **Stability study**

a) Stability after centrifugation

Immediately after fabrication, 1 ml of ME-DEO and ME-SCO samples, placed into 1.5ml micro tubes, were submitted at various centrifugation runs (for 10 minutes at cycles of 3000 to 10000g). The visual aspect for each preparation was observed, and the last centrifugation was used for absorption spectra (AS) studies.

b) Medium term stability

The microemulsions were observed for 180 days at three different temperatures [room temperature (25 ± 1ºC), 4 ± 0,02ºC and -20 ± 1ºC]. Their visual aspect, pH and AS were performed at the day of preparation and after 7, 15, 30, 60, 90 and 180 days. The AmB content was determined by the calculation of the molar extinction coefficient (ε) maximum at 404nm for each AS using a Microsoft Excel Program.

Results and discussion

At the day of preparation all ME were not only clear and stable but also presented a Winsor IV phase that remained after the incorporation of AmB. The AS study shown that both ME-DEOand ME-SCO spectra exhibit three maxima at 365, 384.5 and 409 nm and a shoulder around 347 nm

These spectra are quite similar to those obtained in methanol media, but they are shifted to the right (**Figure 1**). Therefore, the ME-DEO and ME-SCO spectra present a monomeric band that is shifted to a longer wavelength, at 409nm, compared to the AmB monomeric band in methanol, which presents a maxima at 404.5nm. In addition, the dilution study of these preparations shows that the ME systems present a ε_{409} that did not change over the range of concentration tested [5 x 10^{-5}M to 5 x 10^{-8}M (results not shown)].

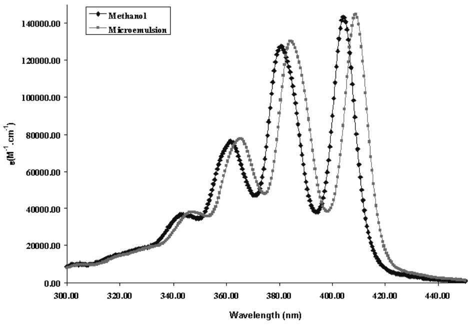

Figure 1 - Absorption spectra for ME-SCO at 5 x 10^{-6}M in methanol and in microemulsion

Concerning the centrifugation stability study, no difference was observed after 1 hour of centrifugation for ME-DEO system, which was charged with 2 x 10^{-3}M of AmB, neither for the visual aspect nor for its spectra. However, the ME-SCO presented not only a yellowish precipitate at the bottom of the tube, but also a reduction of 20% in the ε_{340} band. These results suggest that despite the lower molar % for ME-DEO system, it has a higher load capacity than ME-SCO. This could be due to a better molecular organization for the microemulsion containing sodium deoxycholate [6].

The visual aspect and pH of the systems stayed practically unchanged; a Winsor IV aspect was maintained and no variation on the pH was observed. For systems where the AmB was incorporated, a cloudy solution was observed for products stocked at -20ºC. For products placed at room temperature (RT) or 4ºC, after 7 days, a small precipitate was observed, which increased with the time. This precipitate seems to be a kind of AmB aggregate that did not absorb in a range of 300 to 450 nm. In fact, during the AmB content analysis, a reduction in absorbance observed at the λ_{404} band, but after some time (48hrs) this band was stabilized to a normal absorbance of about 0.805.

The medium-term stability study shows that, in spite of the liquid media and the AmB molecular state existence, both systems are able to protect the AmB against hydrolysis and temperature variation. At -20ºC a decrease in the AmB content was observed only after 3 months for ME-DEO (86.6%) and 6 months for ME-SCO (94.3%). The other temperatures of study (4ºC and RT) maintain the amount of AmB quite stable with some important reduction only after 6 months.

Conclusion

These results suggest that the microemulsions could be excellent carriers for lipophilic molecules such as AmB. In this study we have shown that the AmB-ME is a system where the AmB exists only in a monomeric state, and also they maintain the stability of the drug. Moreover, because of excellent human compatibility and the reported lower toxicity of AmB monomeric form, these systems could become improved new carriers for AmB.

Acknowledgements

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DD-30 CHITOSAN MICROPARTICLES OBTAINED BY SPRAY DRYING

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Introduction

Chitosan (CH) is a natural polysaccharide that origins from the deacetylation of chitin, the second most abundant polymer after cellulose. Chitin is the main material of the exoskeleton of crustaceans, and it can be obtained as a byproduct from the seafood industry. The last decade the applications of chitosan in pharmaceutics have been remarkably increased. Chitosan has been referred as a mucoadhesive polymer and it has been used as efficient absorption enhancer of insulin and salmon calcitonin across nasal mucosal epithelia [1]. It has been shown that CHT also improves the absorption of peptides and proteins across nasal and intestinal epithelia. The enormous advance of genetic engineering and the rDNA technology have been responsible for the production of significant quantities of a wide variety of biologically active peptides and proteins that are therapeutically applicable. Unfortunately, despite the great potential of these new therapeutic drugs only a few of them has received approval by the FDA. This has been, in general, a consequence of the lack of appropriate form of administration, which would allow them to be fully exploited. The peroral route is considered to be the most convenient way of drug application for the patient. The application of peptides through the intestine, however, faces several obstacles. These are the deterioration of the peptide structure by gastric pH and intestinal enzymes, as well as the poor absorption of intact peptide structures through the gut wall [2]. We are currently developing chitosan microparticles obtained by spray drying (SP) and the aim of the investigation is to evaluate the SP technological parameters, which may affect the particle size, particle size distribution, shape, production yield and encapsulation efficiency of the microparticles. Inlet/outlet temperatures, aspirator setting, spray flow and spray concentration/particle sizes were studied. In order to study the dissolution kinetic behavior of the chitosan microparticles and their microencapsulation pattern, a model drug, was taken.

Materials and Methods

The chitosan used in this study were kindly given by Sigma, Bioagro and Dupond. Ibuprofen was a generous gift from BASF, Germany. All other chemicals were of analytical grade. A cutting mill was used to grind the chitosan (Analysenmühle, Janke-Kunkel). A spray dryer (nozzle 0.7 mm) was employed to obtain the microparticles (Büchi modelo B-191, Flawil, Suiza).

- **Characterization of the microparticles**

Particle size

The diameter of the microparticles were obtained by Laser light scattering, employing a *MALVERN Mastersizer X* (MALVERN Instruments Ltd., Worcestershire, GB) with a He-Ne-laser (λ=0.63 nm).

- **Encapsulation of the model drug**

The encapsulation of the drug was checked by differential scanning calorimetry (DSC7, Perkin Elmer).

- **Morphology of the microparticles**

The surface and shape of the microparticles was visualized by scanning electron microscopy (Jeol JSM5300).

- **Dissolution behavior of chitosan microparticles**

Microparticles formulations were incubated in phosphate buffered saline pH 7.2 over 8 hours. The USP basket stirring method was used.

Results

0.7% and 1% CHT concentration and 0.125 µm CHT particle size showed to be the most suitable conditions concerning to the yield of microparticles production. The yield of production of spray dried microparticles varied between 30% and 37%. The SP settings for CHT were 90% aspiration rate, 92% spray flow and 105ºC-inlet temperature.

The microparticles diameter was around 10 µm to 15 µm.

The Differential scanning calorimetry graphs of CHT as a fine powder and as microparticles are very similar,, both show an endothermic signal around 150ºC, which could be related to molecular water from the chitosan.

The scanning electron micrographs of CHT microparticles show spheres with a very smooth surface without any evidence of non-encapsulated drug and its dissolution percentage was about 99.9 % after 8 hours.

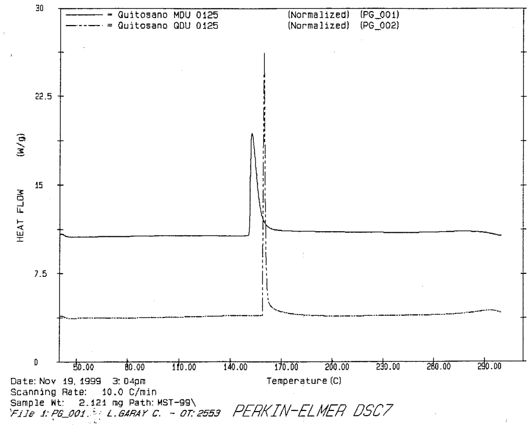

Figure 1. Differential scanning calorimetry graphs of CHT as a fine powder and as microparticles

Conclusions

The SP parameters affected the particle size and yield of both polymers studied. The concentration and the particle size of the CHT dispersion were crucial to obtain CHT microparticles. The dissolution rate of encapsulated drug in the microparticles as well as the electron micrographs showed that CHT spray dried microparticles could be a very promising carrier for the administration of drugs.

Acknowledgements

We thank the CEPEDEQ of the University of Chile for their support.

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DD-31 CHITOSAN-ALGINATE MICROSPHERES PREPARED BY COMPLEX COACERVATION: STUDY OF POLYELECTROLYTE COMPLEX

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Introduction

Microcapsules with an alginate gel core and a polyanion-polycation membrane of polyelectrolyte complex have been widely investigated in connection with various applications [1,2]. Encapsulation of yeast cells in ethanol production, immobilisation of hybridoma cells in production of monoclonal antibodies, encapsulation of insulin producing islets for reversal of diabetes and encapsulation of drugs in sustained release systems represent some examples [3].

Polyelectrolyte complexes are formed by the reaction of a polyelectrolyte with an oppositely charged polyelectrolyte in an aqueous solution. Polysacharides, which have bulky pyranose rings and highly stereoregular configuration in their rigid, linear backbone chains, have frequently been studied [2].

Chitosan is a (1,4) linked 2-amino-2-deoxy-β-D-glucan and can be prepared by the N-deacetylation of chitin. Chitosan has been examined by the pharmaceutical industry due to its potencial use in controlled drug release systems. The cationic character added its reactives groups give to chitosan only properties for use in controlled release [1, 2, 3, 4]. Alginate comprises a linear, unbranched chain of (1,4) linked β-D-mannuronate and α-L-guluronate residues arranged in a blockwise fashion. Alginate has many applications in food technology and its gelation by divalent cation has been widely used [2].

The aim of this work was to investigate the formation of polyelectrolyte complex between chitosan and alginate (CS-AL) and the preparation of chitosan-alginate microspheres.

Materials and Methods

Materials

Chitosan of practical grade with deacetilation degree of 63% (molecular weight 1.4. 10^5) was purchased from Sigma; sodium alginate of high viscosity (molecular weight 3.27. 10^5) was purchased from Henrifarma; calcium chloride and all other chemicals used were of analytical grade.

Methods

- **Characterization of polyelectrolyte complex by viscosimetric and conductimetric analyses**

In order to assess the mass ratio between the polymers to be used in the CS-AL complex formation and the appropriate pH for the medium, viscosimetric and condutimetric analyses were accomplished.

In the viscosimetric analysis a 0.25% chitosan dispersion in 0.1N acetic acid was added into a 0.5% sodium alginate dispersion under stirring. After 30 minutes of stirring, the complex formed was separate by filtration through paper and the viscosity was measured by Selecta (Visco Star L) viscometer. The polyelectrolyte complex was prepared using different volumes of both dispersions and the pH of both dispersions was set at different values (2.8; 3.8; 4.8 and 5.8).

In the conductimetric analyses, an initial volume of 80 mL of 0.01% chitosan dispersion was placed in a flask in contact with the conductimetric cell and samples of 1mL of 0,1% alginate dispersion were added with constant stirring. The resulted conductivity was registered after each addition and stabilization.

- **Infrared analyses of polyelectrolyte complex formation**

The building of CS-AL polylelectrolyte complex was also followed by infrared spectroscopy (Bomem – M Series) of through samples of chitosan, alginate, physical mixtures of polymers and polyelectrolyte complex prepared as KBr disks.

- **Preparation of CS-AL microspheres**

A 0.5% alginate dispersion was dropped through a syringe with a 26 ½ gauge needle. The droplets were pulled off into 50mL of a 0.25% chitosan dispersion containing calcium chloride. After reticulation, the microspheres were washed with water and freeze-dried.

The polymer ratio, the calcium concentration and the contact time of the capsules with chitosan dispersion in the formation and its structures of the microspheres were analyzed.

The resulted microspheres were analyzed to size distribution and morphology by optical (Leica – DM RXA) and scanning electronic (Jeol T330A) microscopy.

Results and Discussion

Viscosity measurements confirmed the polyion complex formation. Figure 1 shows the viscosity of supernatant in the CS-AL mixture as a function of CS-AL ratio and pH of the medium. The optimal pH value for polyelectrolyte complex building was 4.8, providing higher degree of complexation, smaller viscosity of the supernatant. From viscosimetric analysis the optimal CS-AL mass ratio was found to be 2:1.

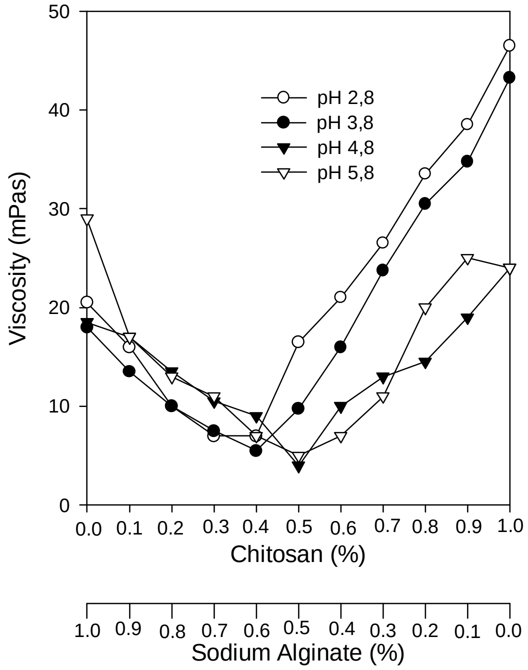

Figure 1**.** Viscosimetric analyses of polyelectrolyte complex formation of chitosan and alginate at different pH values.

The polyelelctrolyte complex formation was confirmed by conductimetric and infrared analyses.

The method used allowed the production CS-AL microspheres showing spherical shape, flat surface and size uniformity.

During microspheres formation, by the contact of the dispersions, the calciums ions diffuse more quickly than the chitosan ones, building a membrane that covers the gelled core [5].

The results show that both calcium ions concentration and contact time of spheres with chitosan dispersion are important for the building and for the structure of the microspheres obtained.

Conclusion

Viscosimetric and conductimetric analyses characterized the CS-AL polyelectrolyte complex formation as well as the optimal polymers mass ratio and pH of the medium for formation of such a complex. By means of the coacervation complex method spherical particles with good morphology and uniform size were obtained.

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DD-32 ETHYLCELLULOSE MICROPARTICLES CONTAINING PRAZIQUANTEL

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Introduction

Modified and particularly sustained delivery can be achieved by the microencapsulation of a drug. As a result of this process, microscopic particles with matricial or reservoir structures can be obtained [1]. Microcapsules as drug delivery system show the advantage to be readily and widely distributed within the gastrointestinal tract, increasing the resorption process and reducing irritant effects on mucosae [2].

Ethylcellulose (EC) has been widely used as wall forming agent, because of its safety and stability. The nature of the EC-film, which is water permeable, but insoluble, offer a wide range of application [3].

The emulsification-solvent evaporation method is usually employed to microencapsulate water insoluble drugs. Typically this method involves the dissolution or suspension of the drug in a solution of the wall forming polymer in a volatile solvent. Since the solvent is water imiscible, an emulsion is building and the solvent can be further evaporated by warming or vacuum. This results in the solidification of the polymeric material involving the drug. The microparticles can then be recovered by filtration or centrifugation, and dried [4].

Praziquantel (PZ) is an acylated derivative of pirazine-isoquinoline. It is active against *Schistosoma mansoni, S. haematobium, S. japonicum, S. intercalatum e S. mattheei.* It shows special importance in the treatment of cerebral cisticercosis [5]. Since PZ has short half life it seems to be a good candidate for microencapsulation processes.

The aim of this work was to prepare and characterize EC-microparticles containing PZ.

Materials

Ethylcellulose (Sigma®), Praziquantel (Henrifarma), Ethyl acetate (Carlo Erba), Tween 80 (Sigma®).

Methods

- **Microparticles preparation**

Microparticles with or without PZ were prepared and analyzed. A typical batch was obtained by dissolving EC (0.67g) in ethyl acetate (20mL) under stirring at 13,000 rpm (Ultra Turrax®) during 5min. PZ in the desired concentration was added and the mixture was stirred for further 2 min. Batches of 10, 20 or 30% PZ in relation to EC mass were prepared. This solution (15 mL) was added dropwise into a 0.4% Tween 80 solution (35 mL), maintaining the stirring during 7 min. The emulsion was placed in a rotate evaporator and the solvent was evaporated during 30 min at 30°C. The resulted suspension was centrifuged at 1,500 rpm during 20 min, washed twice with bidistilled water and centrifuged again. The particles were freeze-dried during 24h.

- **Morphological analyses**

Morphological analyses were carried out by optical microscopy (Carl Zeiss, mod. Jenamed 2) during all steps of the preparation process. Dried samples were suspended in 1% Tween 80 and placed on microscopy slides. SEM (JEOL, mod. JSM T330A) was performed on freeze-dried samples, which were covered with gold.

- **Size distribution**

The size distribution was performed by Leica Qwin software, which captured images from a Leica DMRXA microscope. The Feret´s diameter by 0°, i.e. the greater dimension of each particle in the horizontal direction was measured [6]. Randomically choosed fields were measured, without repeating fields and particles.

- **Calibration curve of PZ**

The calibration curve for PZ was assessed in acetonitrile/water (3:2), the mobile phase of the HPLC method used. Dilutions from of 0.3 to 10 µg/mL were prepared, filtered and analyzed in triplicates.

- **Entrapment efficiency**

The microparticles (0.02g) were dissolved in 5ml of mobile phase by stirring at 19,000 rpm during 1 min, filtered through acetate membrane (Millipore, 0.45 µm pore) and, after adequate dilution, analyzed by a HPLC method, not interfered by EC. The chromatographic conditions used were: isocratic elution, mobile phase acetonitrile:water (3:2), flow rate 1 mL/min, λ = 262nm, injection volume = 100µL, column temperature ~30 °C, stationary phase: Lichrospher 100RP-18, 5 µm.

- ***In vitro* delivery of PZ**

The *in vitro* delivery of PZ was performed by an adapted USP apparatus 3 for using with spectrophotometric cubets [7] on batches of 10, 20, and 30% PZ. Sink conditions were observed. The experimental conditions are: temperature of 37ºC, cellulose acetate membrane, medium: aqueous sodium laurilsulfate 2mg/mL, λ = 262nm.

The amount of drug delivered was quantified by means of the previously described HPLC method

Results

- **Morphological analyses**

The microparticles showed spherical shape in all steps of the production process. As SEM photomicrographies show, microparticles are of rough surface and great morphological homogeneity.

- **Size distribution**

The size distribution ranged from 0.5 to 10µm, the majority of the particles being between 0.5µm and 4.5µm for inert particles. Drug containing particles show sizes ranging from 2.5µm to 4.5µm.

- **Entrapment efficiency**

The entrapment efficiency was measured by the ratio between actual and theoretical amount of encapsulated drug. The entrapment efficiency was 51.02% for batches with theoretical amount of 10% PZ, 41.38% for 20% PZ and 52.68% for 30% PZ.

- ***In vitro* delivery of PZ**

After 12 h the amount of PZ delivered was 36.77% 57.05% and 63.39% for batches of 10, 20, and 30% PZ, respectively.

Conclusion

The method of emulsification-solvent evaporation is able for the preparation of EC-microparticles containing PZ. The entrapment efficiency was good and the microparticulate system controlled the drug delivery, as showed by *in vitro* tests.

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DD-33 3-[1,2-DIHYDRO-5-(5-HYDROXY-1H-INDOL-3-YL)-2-OXO-3H-PYRROL-3-YLID-ENE]-1,3-DIHYDRO-2H-INDOL-2-ONE ENCAPSULATED IN A POLY-CAPROLACTONE MICROSPHERES: CYTOTOXICITY AND ANTITUMORAL ACTIVITIES

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Introduction

3-[1,2-Dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ylidene]-1,3-dihydro-2H-indol-2-one (violacein) (Figure 1) is effective against Gram-positive and Gram-negative bacteria.

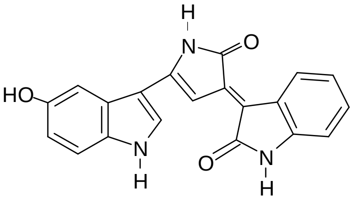


Figure 1. Violacein

Antimycobacterial, trypanocide, tumoricide, antiulcerogenic, antioxidant, antiviral [1,2] and antileishmaniosis activity were also studied [3]. The antitumoral activity and its cytotoxicity were exerted through apoptosis [4]. The biodegradable microspheres and nanospheres involved in a controlled drug release have been extensively studied in the last years. These systems offer many advantages when compared to a conventional dosage systems as the progressive delivering of bioactive agents [5]. Polycaprolactone is one of the most common material for the microspheres formation in encapsulation methods [6].

Aims

The aim of this study was to prepare the polycaprolactone microspheres with encapsulated violacein and to study some of its biological properties.

Methodology

The biosynthesis and purification of violacein were performed as previously published [7]. The polycaprolactone microspheres with and without violacein incorporation were obtained by dissolving in ethyl acetate with an emulsion formation in water in the presence of a surfactant. After organic solvent evaporation the microspheres were formed.

The temperature (50°C) and surfactant concentration (over 2%) were important parameter for avoiding the coagulation processes. Microspheres in a scanning electronic microscope (topography) observation exhibited diameters from 2 to 10 μm, with round forms and smooth surfaces (Figure 2). The violacein incorporation was attested by fluorescence microscope and its absorbance at 575 nm (Figure 3). Human leukemia cells (HL60) were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin in a humidified atmosphere at 37ºC in 5% CO_2. Cells were seeded (3 x 10^5 cells/mL) in 96 wells plates and 35 mm plates, incubated with violacein, violacein/ microspheres in different concentrations during 72 hours. Cell viability was determined by MTT reduction test as described elsewhere [8]. V79 Chinese hamster lung fibroblasts were routinely grown in DMEM containing antibiotics and supplemented with 10% fetal calf serum [4]. For the MTT, NAC and NRU assays, 24 well tissue culture plates were inoculated with 3 x 10^4 V79 cells/ml and incubated 48 h at 37°C. The cells were incubated for 24 h with 2-30 μM violacein and violacein/ microspheres.

Apoptosis was evaluated by morphological criteria as previously described [4]. After treatment the cells were fixed in ethanol/ acetic acid (3:1) and the slides were submitted to the Feulgen reaction, and viewed at 1000 X magnification with the aid of an Olympus microscope. The apoptotic cells were defined as those exhibiting the characteristic feature of cell shrinkage, nuclear condensation, and the formation of membrane-bound apoptotic bodies. Lethality by brine shrimp assay [9] was also evaluated.

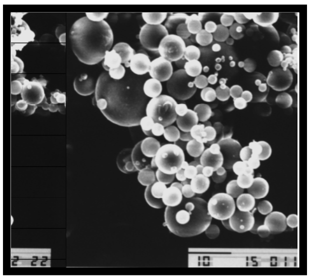
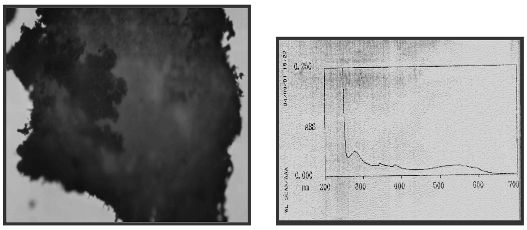


Figure 2. SEM of microsphere of polycaprolactone (SEI,1000x, 15 kV).



(a) (b)

Figure 3. Fluorescence (a) and absorbance (b) of violacein in the microspheres.

Results and Discussion

Using the MTT tetrazolium reduction test on V79 cells, the IC_{50} for the violacein alone were less than 1 μM. For the MTT (IC_{50} 7 μM), NAC (IC_{50} 7 μM) and NRU (IC_{50} 12 μM) assays, 24 well tissue culture plates were inoculated with 3 x 10^4 cells/ml and incubated 48 h at 37^oC. The cells were incubated for 24 h with 2-30 μM violacein and violacein/ microspheres. Cytotoxicity in violacein/ microsphere slightly increased. Moreover, using the Feulgen reaction violacein and the derivatives were found to trigger apoptosis in HL60 cells. The morphological changes in the nuclei of these cells included chromatin condensation and formation of apoptotic bodies. In the violacein/microspheres the results were more pronounced than with violacein alone. Similar results with the lethality by the eggs of brine shrimp assay with both violacein and violacein/microsphere were found.

These results provided a basis for the potential therapeutic application of co-formulation of violacein with microsphere in cancer therapy

Support from FAPESP is acknowledged.

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DD-34 THE USE OF MTDSC AND GPC TO STUDY THE DEGRADATION BEHAVIOUR OF PLA MICROSPHERES

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Introduction

Poly (d, l – lactide) PLA is a copolymer of two stereoisomers, D- and L-lactic acid. Due to its amorphous nature it degrades much faster than the homopolymers of D- or L- Lactic acid, which have crystalline structures [1]. PLA has been used for the controlled release of pharmaceutical drugs because of its biocompapatible and biodegradable properties. Drugs encapsulated within these microspheres have been used as injectable controlled delivery release systems, which do not require removal after the drug has been depleted [2]. PLA degrades into natural by-products of the body and is excreted as lactic acid. It is believed that these microspheres degrade homogeneously by bulk erosion, although studies have also shown heterogeneous degradation of large particles [3]. The objective of this study was to determine whether these PLA microspheres undergo homogeneous or heterogeneous degradation and investigate the effect of 0.1*M* phosphate buffer saline (PBS) on the glass transition temperature of PLA microspheres which are dehydrated (dried in oven at 37 C for 5 days) and hydrated (un-dried analysed straight from PBS).

Experimental methods

- **Materials**

The following materials were received from commercial suppliers: Sigma, Poole, UK supplied PLA Mw 106,000. BDH, Poole supplied polyvinyl alcohol Mw 22,000, potassium dihydrogen orthophosphate and sodium hydroxide solution. PLA Microspheres with a mean volume median diameter of 7.7 µm were prepared using a solvent evaporation technique. 500 mg of PLA was dissolved in 10 mL of dichloromethane. The solution was poured into 125 mL of 2% w/v polyvinyl alcohol solution and homogenised for 2 minutes at 8000 rpm. The resulting emulsion was stirred continuously for 20 hours with an overhead stirrer at 500 rpm. Once microspheres had formed, they were incubated in 0.1*M* PBS pH 7.4 and agitated at 40 rpm in a water-bath at 37°C. At predetermined intervals the microspheres were isolated from PBS and analysed. The surface morphology was observed by scanning electron microscopy (SEM) (Philips XL20) Particle size was measured by laser diffraction (Malvern Mastersizer SX). Mass loss was determined gravimetrically; weight loss was determined by gel permeation chromatography (GPC). Modulated temperature differential scanning calorimetry (MTDSC) studies were also performed to analyse PLA's glass transition temperature when hydrated (TgH) and dehydrated (TgD) (TA Instruments 2920).

Results and discussion

Table 1.
Effect of degradation on mass, molecular weight and glass transition of PLA incubated at 37ºC and pH7.4 (mean ± SD = 3).

Day	Mass Loss (%)	Molecular Weight (Mw)	Poly dispersity (Pd)	Tg H (°C)	Tg D (°C)
0	0	112100 ±2185	1.9	55.33 ±0.42	55.33 ±0.42
10	13.69 ±3.3	95400 ±2600	1.9	51.42 ±0.56	53.59 ±0.25
20	11.74 ±4.0	86600 ±1990	2.2	40.88 ±0.04	50.81 ±0.42
30	15.81 ±2.7	82990 ±2365	1.7	40.99 ±0.26	51.63 ±1.41
40	17.17 ±3.0	75700 ±1900	1.8	40.81 ±0.33	51.91 ±1.82
50	19.57 ±1.4	75700 ±3300	1.8	-	-
60	23.60 ±8.8	75400 ±2100	1.6	40.61 ±0.38	51.46 ±0.19
70	30.65 ±1.7	68700 ±2100	1.6	40.48 ±0.67	51.23 ±0.28
80	29.93 ±4.4	67700 ±1500	1.6	42.50 ±0.37	51.72 ±0.79
90	30.13 ±3.3	65000 ±2200	1.5	42.71 ±0.34	51.56 ±0.50
100	29.29 ±5.2	61000 ±490	1.5	41.30 ±1.01	51.72 ±0.37

As shown in table 1 as that the length of incubation time increases the microspheres mass loss and weight loss increased, whilst the glass transition of the hydrated and dehydrated micropsheres decreased to an extent. The initial glass transition (T_g) of PLA was approximately 55°C. By day 100 the hydrated microspheres T_g had dropped to approximately 42°C. The decrease in T_g became virtually constant as from day 20, which could imply there is a limit to the plasticsiation effect of PBS on the PLA microspheres. The dehydrated microspheres on the other hand showed a small decrease in T_g though the effect was less forthe hydrated PLA microspheres. The T_g of the dehydrated PLA microspheres dropped to approximately 52°C by day 100. The T_g of the dehydrated PLA microspheres became constant from day 20. The T_g temperature did not revert back to the initial T_g temperature of 55°C after drying. This may be an indication of the onset of degradation. Both hydrated and dehydrated PLA microspheres were in the glassy state since the T_g temperature did not drop below 37°C.

In the glassy sate the mobility of the chains are restricted, so high molecular weight PLA microspheres would have slow diffusion of PBS.

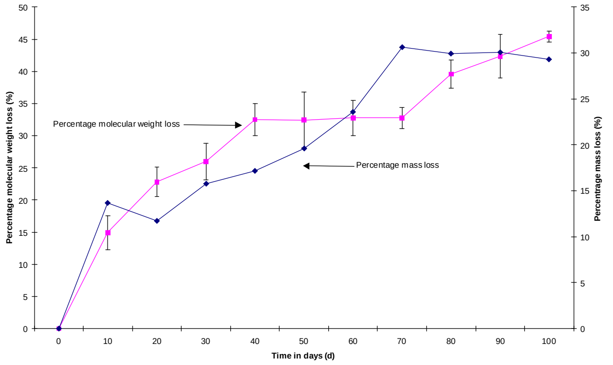

Figure 1. Percentage mass and molecular weight loss changes, of PLA microspheres in 0.1M PBS pH7.4 at 37 ^{0}C (mean ± SD = 3).

In Figure 1 there is an apparent relationship between the percentage molecular weight loss and percentage mass loss. As percentage molecular weight loss increased so did the percentage mass loss. By the end of the 100 days the percentage mass had dropped by 30% and the molecular weight had dropped by 45%. This indicates molecular weight loss occurs more rapidly than mass loss in PLA microspheres. Figure 2 shows the molecular weight change with time. The PLA microspheres show an apparent triphasic degradation profile. An initial accelerated phase of 40 days with a hydrolysis rate of 4.7×10^{-3} d^{-1}, followed by a relatively stationary phase with a hydrolysis rate of 8.9×10^{-5} d^{-1} and a final but slower accelerated phase with a hydrolysis rate of 2.3×10^{-3} d^{-1}at day 60. This profile could be interpreted as rapid surface erosion, followed by slow penetration of buffer into the microspheres followed by fast bond cleavage. SEM studies revealed microspheres retained their original shape and had a slightly rough surface with no pores.

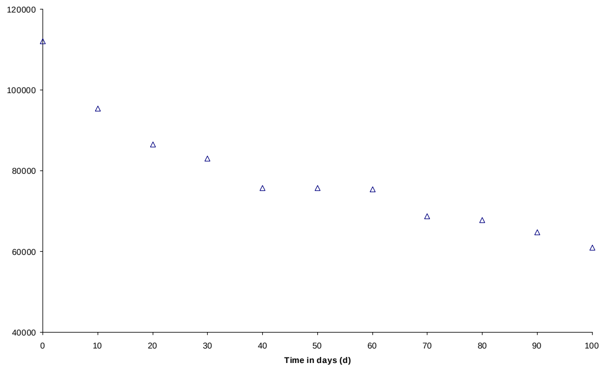

Figure 2. The change in molecular weight of PLA microspheres with time (mean ± SD = 3).

Conclusion

GPC studies indicate that there may be more than one phase of degradation of PLA microspheres. Work done by Reich [3] suggests several phases exist and there is some evidence of this in the present study The MDSC studies show there is a limit to PLA plasticisation in PBS over100 days. SEM and GPC studies show PLA microspheres maintain their original shape, but steadily lose mass during degradation, which implies these microspheres are undergoing homogeneous degradation.

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DD-35 COATED IBUPROFEN PELLETS: EFFECT OF COMPACTION ON DRUG RELEASE

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Introduction

The design and development of multi-unit dosage formulations (containing microparticles) in the form of compressed tablets, rather than gelatin capsules, have become increasingly important. Hard gelatin capsules are very elegant dosage forms, but have the disadvantage of higher production cost and lower production rate when compared to compressed tablets.
When sustained release tablets containing coated or uncoated microparticles are manufactured, it is often desirable to produce compacts that disintegrate into many subunits soon after the ingestion to attain more uniform concentrations of active substances in the body [1]. The difficulty involved in such an approach is that films can fracture upon compaction and produce a large difference in the release characteristic from the coated pellet before and after compaction. The manufacturing method of microparticles, the type and amount of coating agent, size of the microparticles, selection of the external additive, and the rate and magnitude of the pressure applied must be carefully considered to maintain the desired drug release properties [2].

The goals of this study were to investigate the effect of pellet particle size, amount of coating agent and the magnitude of the pressure applied for tabletting.
Ibuprofen was used as model compound because it is a non-steroidal anti-inflammatory agent. The oral dose is 1.2 to 1.8 g. increased to 2.4 g. daily. The most frequent adverse effects occurring with ibuprofen are gastro-intestinal disturbances. Ibuprofen has a half-life of about 1.8 to 2.0 hours, so that it is necessary high doses divided in short time intervals. The adverse effects are dose dependent [3]. Those characteristics make the controlled release tablets a suitable choice for this anti-inflammatory agent.

Materials And Methods

Ibuprofen used in this study were kindly given by BASF, Germany. Eudragit^{TM} S-100, Eudragit^{TM} RL30D and Eudragit^{TM} RS 30D were a generous gifts from Röhm Pharma, Germany. All other chemicals were of analytical grade.

- **Formulation and process for pellets**

Pellets were manufactured using a *pellet pan* (Erweka GTE/UG, Germany) and a *manual spray gun*. The pellet formulation consisted in ibuprofen 95.6% and Eudragit^{TM} S-100 4.4 %. Pellets having mesh cuts of: 420-590 µm (40/30 mesh), 300-420 µm (50/40 mesh), 250-300 µm (60/50 mesh) were studied.

- **Coating formulation and process**

Microparticles were manufactured using a *coating pan* (Erweka, Germany) and a *spray gun* (Binks Bullows, England). A pump (MasterFlex, USA) was used to deliver the feed of the coating suspension. The microparticles consisted in: pellets 94-92-90 % and Eudragit^{TM} RL/RS 30D 6-8-10 %.

- **Particle size**

The diameter of the microparticles was obtained by sieving method (ASTM sieves).

- **Tablet formulation**

Tablets consisted in: microparticles 75 %, spray dried lactose 20 %, Explotab^{TM} 4 % and magnesium stearate 1 %.

- **Compaction studies**

Compaction studies were done on an instrumented eccentric tablet press, Hanseaten Wilhelm Fette (Germany). The press was set at 10-15-20-25 kN ± 0.5 compaction force.

- **Dissolution rate of the pellets, microparticles and tablets**

Pellets were incubated in phosphate buffered pH 7.2 ± 0.05 over 2 hours. Microparticles and tablets were incubated in phosphate buffered pH 7.2 ± 0.05 over 8 hours. The USP basket stirring method was used. Ibuprofen was evaluated by spectrophotometry at λ=221 nm.

Results

- **Pellets dissolution rate**

The ibuprofen dissolution percentage was 100% after 2 hours for all the pellet sizes.

- **Microparticles dissolution rate**

The ibuprofen dissolution percentage was depending on the coated pellet size. After 6 and 8 hours, 100% of the ibuprofen was dissolved when the coated pellet size corresponded to 40/30 mesh coated with 6 and 8 % of Eudragit^{TM} RL/RS 30D, respectively. All the other coated pellet sizes manufactured in this study were not able to delay release of the ibuprofen. According to these results the 40/30 mesh 8% coating was chosen to continue with the manufacturing of the tablets.

- **Influence of compressional force over tablet dissolution rate**

When the tablets were compressed the deformation of the material showed a plastic behavior over 10 to 25 kN. The dissolution test of the tablets pressed at 10 and 15 kN showed a similar profile than the microparticles, proving there was no breakdown of the coating material. It is worth it to point out that after the dissolution test those tablets pressed at 15 kN showed into the stirring basket a remaining solid core which did not dissolve.

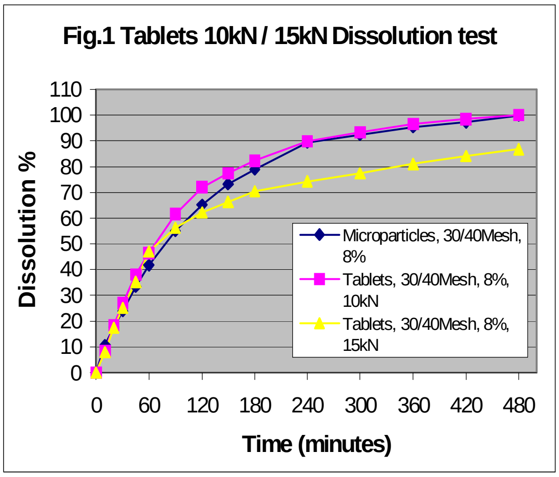

Figure 1. Dissolution test.

Conclusions

This investigation showed that films manufactured from Eudragit^{TM} RL30D and Eudragit^{TM} RS30D, are suitable for the controlled release of ibuprofen from 420-590 μm pellets. These films have the appropriate mechanical properties to withstand compaction forces without rupturing. The controlled release properties of the compacted-coated pellets were not lost during the process.

Acknowledgements

We thank the CEPEDEQ of the University of Chile for their support.

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DD-36 OPTIMIZATION OF CHITOSAN MICROSPHERES PREPARED BY SPRAY DRYING AS INTRANASAL DELIVERY SYSTEMS

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Introduction

In recent years the nasal route has received a great deal of attention as a convenient method for the systemic administration of drugs [5]. Nasal route has certain advantages concerning the absorption of peptides and proteins into the systemic circulation. It could provide a rapid pharmacokinetic profile in comparison with subcutaneous route. Furthermore, intranasal administration can improve bioavailability, security and metabolic efficacy (ILLUM & DAVIS, 1992; PERESWETOFF-MORATH, 1998). In addition, it is a no invasive alternative method for drug delivery (MITRA et al., 2000). The subepithelial layer has a high vascularization and venous blood passes directly into the systemic circulation avoiding thereby first-pass metabolism in the liver [8].

Chitosan is one of the most abundant polysaccharide in nature, which is a product of chitin found in the cell walls of various fungi, crustaceans and insects [7]. Sustained release chitosan microspheres prepared by spray drying methods were previously described [4]. FELT et al. [3] mentioned that chitosan is one bioadhesive and biodegradable polysaccharide with potential application as a drug carrier for many administration routes [9,10].

Objectives

The aim of this work was to study the influence of spray drying parameters (e.g., flow rate, chitosan concentration, and inlet temperature) on particle size, morphology, size distribution, yield and water content in microparticles intended nasal delivery.

Methodology

Two solutions of chitosan 1% and 2% (w/v), in acetic acid 2% were spray dried (Spray Dryer Type B-191, BÜCHI) with a 0.7mm nozzle and co-current flow under inlet temperature varying from 140°C to 180°C and feed rate from 5 to 7mL/min. The spraying pressure was kept constant at 600mmHg. All microspheres batches were checked for shape and micromeritic analysis by optical microscopy (LEITZ BIOmed-leica) using 40X lens. 500 particles suspended in mineral oil were observed using the Ferret's diameter. The residual water content of microparticles were determined by loss of weight (FARMACOPÉIA BRASILEIRA, [2]), using 300mg of sample. Each analysis was repeated three times.

Results And Discussion

The best conditions to prepare microspheres were spraying 1% chitosan solutions with feed rate 5mL/min at 180°C (yield: 39.54% ± 1.53). ANOVA of ponderal yields showed that chitosan concentration is the major factor that influences the results ($p = 0.06$). Interactions between the flow rate and temperature ($p = 0.02$) as well as flow rate and chitosan concentration ($p = 0.001$) were also observed.

The moisture content of chitosan microspheres prepared with feed rate 5mL/min is presented in Table 1. The results indicated that a lower feed rate in association with higher temperatures lead to a decrease in residual moisture.

Table 1
Moisture content of chitosan microspheres obtained from 1% and 2% (w/v) polymer solutions.

Temperature	1%	2%
140°C	14,20%	15,10%
160°C	12,12%	11,60%
180°C	11,68%	11,70%

Microscopy showed that most particles presented spherical shape with an unimodal population of mean diameter 5µm. There was no apparent increase in particle size when the flow rate was reduced from 7 to 5 ml/min and the temperature changed from 140°C to 180°C, particle size characteristics remained almost unchanged as shown in Figure1.

Microspheres for nasal drug delivery studies usually present a diameter around 45µm (PERESWETOFF-

MORATH, 1998) in the swollen state and 1-10µm in the dried state [6].

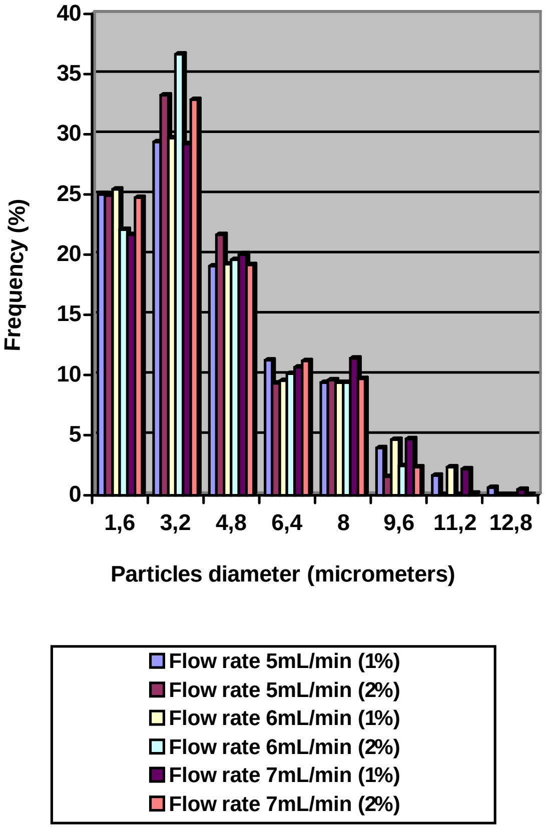

Figure 1: Microscopic analysis of the particles size of chitosan at different flow rates.

Conclusion

According to the results, the spray-drying conditions for chitosan microspheres preparation (the size of nozzle, feeding flow rate, inlet temperature, concentration of chitosan solutions) influenced the ponderal yields of microparticles. In general, 1% chitosan solutions lead to better results. In this case, the yield increases with inlet temperature.

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DD-37 PRELIMINAR INVESTIGATION ON THE POTENTIAL OF MODIFIED CASEIN AS ADJUVANT POLYMER FOR A VACCINE DELIVERY SYSTEM

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Introduction

Adjuvants are substances added to the immunizing antigen in order to enhance the host immune response by augmenting the availability of the antigen to the antigen presenting cell (APC), extending the persistence of antigen in tissues, or stimulating the APC directly. The main problem with adjuvants is that the most potent ones tend to have strong inflammatory properties and it is not clear whether these inflammatory properties can be separated from adjuvanticity.

Vaccine formulations involving micro-encapsulation of antigens have received considerable attention due to the potential for protecting antigens and controlling their rate of release. A number of biodegradable polymers have been investigated focusing such aims. Therefore, there is an urgent need for the development of both improved adjuvants and delivery systems, which could be potents and safes.

Our purpose is to investigate the potential of modified casein as a safe and efficient adjuvant polymer for vaccine delivery systems.

Experimental methods

- **Materials**

Freund's incomplete adjuvant (FIA) Difco Laboratories. Bovine casein (Mundial Química). Acetic acid, acetic anhydride, chloridric acid, TRIS (hydroxymethyl) aminomethane (Synth). PEG 400 and PEG 4000 (Sigma). Sodium chloride (Vetec).

- **Preparation of modified casein [1]**

3.8mL of acetic anhydride were dropped on a beaker under agitation containing 500mL of commercial casein **(COMCAS)** dispersed into TRIS/HCl buffer (10%w/v); pH 7 maintained by NaOH addition. After 2 hours, 26.7mL of same anhydride were dropped on the beaker until pH 3.7, following 2 hours of agitation. Such material was named acetic casein **(ACETICCAS).**

Simultaneously, casein dispersion precipitated by HCl instead of acetic anhydride was prepared, and named **HCLCAS.**

- **Immunogenicity pilot assay (IPA) [2]**

Seven groups composed by one rabbit were subcutaneously immunized, being the group 1 as control and the others as dose groups. Each dose groups was immunized at days 1 and 30 with 1mg/mL (1mL) of **COMCAS** (group 2), **COMCAS+FIA** (group 3), **ACETICCAS** (group 4), **ACETICCAS+FIA** (group 5), **HCLCAS** (group 6), and **HCLCAS+FIA** (group 7). The polymers were dispersed in PBS and emulsified with FIA (50:50) immediately before SC injected. Blood samples were withdrawn at days 1, 15, 30, 48 and 63 in order to coagulate. Conventional indirect ELISA assayed antibody developed against these polymers (with or without FIA).

- **Preparation of microparticles**

Casein microparticles (CAS/MP) were prepared by simple coacervation in aqueous medium, which were shared in three batches in accordance with the polymers (**COMCAS**, **ACETICCAS** and **HCLCAS**), composed by five formulations each one, where theirs variation were fixed for each batch equally (variations: PEG 400, PEG 4000 and NaCl).

- **Physicochemical characterization**

Differential scanning calorimetry (DSC) and thermogravimetrical analyses (TGA) of microparticles were carried out using a DSC-50 and TGA-50 (Shimadzu), respectively.

- **Morphological characterization**

Morphological properties of CAS/MP were evaluated by scanning electron microscopy (SEM) using a Jeol instrument (JSM-T 330A).

Results and discussion

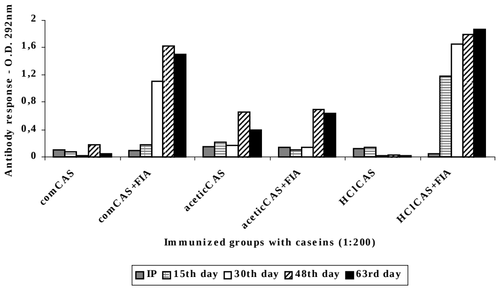

Figure 1. Antibody responses of immunized rabbits with different casein polymers with or without FIA.

Data from IPA displayed that at absence of FIA, **ACETIC CAS** is probably more immunogenic than **COMCAS** and **HCLCAS** when subcutaneously injected in rabbits (see secondary response profiles at Figure 1), this could be due to evoke of immune response, after booster, for a “known“ antigen; however, more hydrophilic. By the way, **ACETICCAS** could be potentially able to expose more efficiently its contact residues on surface of the protein structure due to its lower hydrophobicity. Hence, its epitopes would be more accessible to antibodies.

On the other hand, immunized groups with these same emulsified polymers did not develop such behavior as can be seen at Figure 1. Thus, how to explain why did not the more immunogenic polymer **(ACETICCAS)** present augmented response when emulsified and exposed for a long period related to the others at same conditions? It could be ascribed, in spite of the facts, to **ACETICCAS** being able to evoke secondary response, the magnitude of its response did not increase at FIA presence. This suggests that the modified polymer by acylation should probably present the property of modulating efficiently the development of immune response independent of FIA adjuvanticity mechanism.

Besides, another data from IPA displayed that antibodies developed by immunized groups with **ACETICCAS** and **ACETICCAS+FIA** would not be able to recognize neither **COMCAS** nor **HCLCAS**, indicating that exists a rich possibility of the immune response developed to **ACETICCAS** to be also specific.

Simultaneously, the obtained CAS/MP were evaluated by thermal analyses.

In general, DSC and TGA curves referring to CAS/MP which compose the three batches, suggest that variations on the same batch have lightly affected enthalpic and thermogravimetric behavior (data not presented).

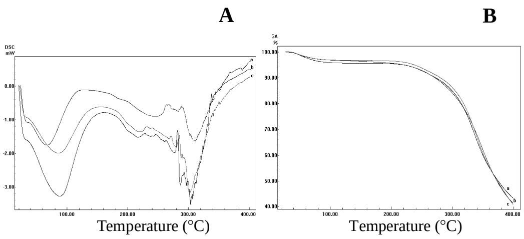

Figure 2. DSC (A) and TGA (B) curves of CAS/MP containing NaCl and PEG 400 prepared from **COMCAS** (a), **ACETICCAS** (b), and **HCLCAS** (c).

According to Figure 2, CAS/MP containing NaCl and PEG 400 have presented an increase of enthalpy, which could be ascribed to the raise of system compactation as result of ionic strength increased [3]. This should lead to the raise of surface tensile between protein-water, consequently raising hydrophobic interactions.

Conclusions

- **ACETICCAS** is probably more immunogenic than **COMCAS** and **HCLCAS**
- **ACETICCAS** should possibly modulate the development of immune response of a specific manner independent of FIA adjuvanticity mechanism
- Casein-PEG blend might occur during CAS/MP preparation
- Formulations containing NaCl and PEG 400 have presented potentially more compacted polymeric structure.

Acknowledments

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DD-38 FORMULATION STUDY OF SUSTAINED RELEASE CARBAMAZEPINE MICROPARTICLES

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Introduction

Carbamazepine (CBZ) is an anticonvulsant drug used for the treatment of trigeminal neuralgia and epilepsy. The usually adult dose is between 600-1200 mg/day. It is, usually, 3-4 divided doses [1]. In order to reduce the incidence of adverse effects related to the carbamazepine plasma concentration fluctuations, sustained-release cellulose acetate butirate (CAB) microparticles has been developed. The aim of this work was to evaluate the influence of two formulations parameters, the polymer molecular weight and the addition of polyethylene glycol 1500 (PEG 1500) in CAB microparticles formulation on the particle mean diameter and CBZ release rate.

Methods

• Microparticles preparation

The microparticles were prepared by the emulsion-solvent evaporation method [2]. A polymeric solution of CAB or CAB/PEG mixture in acetone (100 ml) containing CBZ was emulsified in mineral oil (500 ml) containing 2% (w/v) Span 80 using a magnetic stirrer. The emulsion was continuously stirred for 24 h at room temperature to allow the acetone evaporation from the internal phase. The produced microparticles were filtered, washed with n-hexane and dried under reduced pressure. The drug to polymer ratio was 1:2 for all formulations. The polymer concentration in the internal phase was 5.0 % (w/v). When CAB/PEG 1500 mixture was employed, the entire concentration of polymer in the internal phase was maintained, but the CAB to PEG 1500 ratio was 9:1. The formulations parameters selected in this study were the CAB polymer molecular weight (MW 30000 or MW 70000) and the addition of PEG 1500 in the internal phase of the emulsion (with or without). The tested formulations can be visualized in Table 1.

• Microparticles characterization

The encapsulation efficiency and the drug content (mg/100mg microparticles) were calculated after HPLC analysis of the solutions obtained by CBZ extraction from an amount of exactly weighted microparticles with acetonitrile. The optimum extraction time was previously established as two hours. The HPLC analysis was performed using the following conditions: mobile phase, acetonitrile/water, (45/55, v/v); flow, 1.5 ml/min; column, Supelcosil LC-18; detection, UV 283 nm. The encapsulation efficiency of CBZ was calculated as the difference between the amount of drug initially added to the formulation and the amount found in the microparticles after HPLC analysis.
The mean size of particles was determined using two different methods. When particles were larger than 500μm the measures were performed using a micrometer device. Smaller particle size was measure using an optical microscope calibrated with a stage micrometer scale.

• *In vitro* release study

Carbamazepine release was evaluated using the USP XXIII paddle stirrer dissolution apparatus rotated at 75 rev./min. A 1% (w/v) sodium lauryl sulphate solution maintained at 37 ± 0.5°C was employed as release medium. Microparticles equivalent to 200 mg of CBZ were filled in hard gelatin capsules and added to the release medium. Samples were taken at 30 min intervals for 12 hours and assayed spectrophotometrically for CBZ content at 284 nm.

Results and Discussion

The encapsulation efficiency and CBZ content in the microparticles obtained by emulsion solvent evaporation method using the conditions described above were around 65% and 20% (w/w) for the tested formulations, respectively (Table 2). *In vitro* dissolution profiles of CBZ showed a faster drug release for microparticles prepared with CAB MW 30000 and with addition of PEG 1500 in the internal phase of the emulsion (Figure 1a). On the contrary, microparticles prepared with CAB MW 70000 appears to lead to a very slow release rate of the drug (Figure 1b). This result can be partially explained by the larger particles produced by using this polymer.

Table 1
CBZ microparticles formulations

Formulation	1	2	3	4
Carbamazepine (g)	2.5	2.5	2.5	2.5
CAB MW 30000 (g)	5.0		4.5	
CAB MW 70000 (g)		5.0		4.5
5 % (w/v) PEG 1500 solution (ml)			10.0	10.0
Acetone (ml) qs	100	100	100	100
Span 80 (g)	10.0	10.0	10.0	10.0
Mineral oil (ml)	500	500	500	500

In fact, the CAB 70000 acetone solution showed a higher viscosity than CAB 30000 acetone solution and its employment as polymer in the internal phase of the emulsion leaded to the production of greater particles.

Table 2
Results obtained from drug loading and particle size determinations

	Carbamazepine		MD^{c} (µm) (n=3)
	%^{a}	mg/100mg^{b}	
1	70.39	23.22	294.3 ± 77.9
	72.12	23.94	305.2 ± 77.4
	73.65	24.58	301.3 ± 75.7
2	65.01	21.78	817.3 ± 159.2
	61.21	20.37	1194.4 ± 245.9
	56.21	18.17	577.6 ± 65.5
3	69.72	23.24	335.6 ± 99.6
	65.89	22.02	490.6 ± 94.1
	74.63	24.85	458.3 ± 103.9
4	64.05	21.37	943.0 ± 134.9
	60.01	19.96	720. 6 ± 114.2
	62.52	20.86	677.6 ± 68.4

^{a} Encapsulation efficiency, ^{b} Drug content and ^{c} Particle size

On the other hand, when the particles were produced using CAB polymer with the same molecular weight, the addition of PEG 1500 does not seem to affect the particle size instead it cause a increase of the drug release rate. These results show the improvement of the microparticles delivery capacity provided by the PEG 1500 addition.

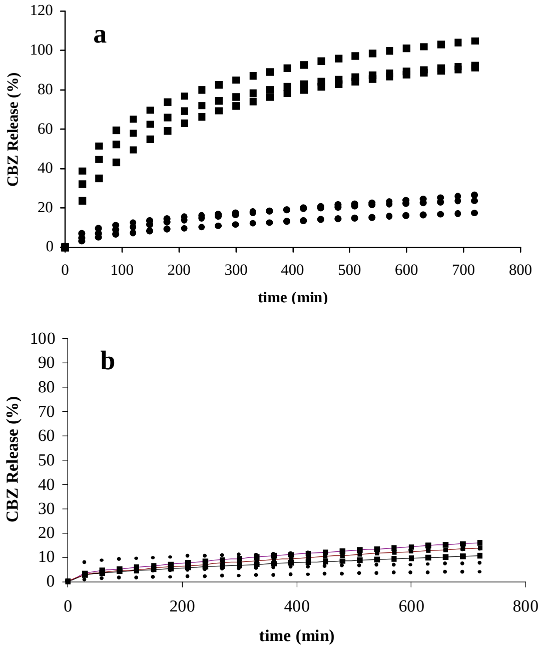

Figure 1 CBZ release profiles obtained from microparticles prepared from: (a) CAB MW 30.000 and (b) CAB MW 70.000. (●)without PEG 1500 and (■) with PEG 1500.

Conclusion

It may be concluded that the employ of CAB 30000 polymer and the addition of polyethylene glycol in the formulations appear to favor the preparation of a more reasonable sustained release microparticles to oral administration.

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DD-39 CHITOSAN-ALGINATE AS MULTIPARTICULATE MATRICES

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Introduction

Natural or synthetic polymers have being used as matrix systems in the encapsulation procedure to entrap drugs, proteins, enzymes, microbiological entities, plant and animal cells. The physicochemical characteristics of such matrix in dried gel form may have effects on the delivery systems of the above active materials entrapped in the systems.

Microencapsulation of microbiological entities using non-toxic materials has been long established [1]. The obtaining of pH-sensitive particles using alginates are widely used for microencapsulation due to their simple and rapid gel formation with divalent metal ions such as Ca^{2+}(2), a method of microencapsulation which is not harmful to live organisms [3].

Alginate microparticle can be coated with an alginate-chitosan membrane (ALG-CHI) by ionic interaction during the preparation. It changes the alginate microparticle characteristics and can sustain the release of an active material in an environment with basic pH. Besides, this membrane can be helpful in the protection of the encapsulated material.

The objective of this work was investigated the preparation of microparticles as encapsulation matrices by reacting the polysaccharide alginate with chitosan to attain a prolonged or pH-dependent release of an biological material incorporated into the microparticles.

Experimental methods

- **Materials**

Sodium alginate of medium viscosity (4000cps for a 2% solution at 25°C) was obtained from SKW-Biosystems, named Saltialgine 1100x. Chitosan was of low molecular weight with a viscosity of 10cps, measured at a concentration of 0.5% (w/w) in HCl, pH 5.0, at 25°C was also used. All others reagents were analytical grade.

- **Production of alginate matrix**

Two different methods to prepare chitosan-alginate microparticles were evaluated. In the first one, calcium alginate gel microparticles were first formed by atomizing a 2% w/w solution of sodium alginate into a 0.1M calcium chloride solution at 20°C, followed by a chitosan-alginate membrane forming stage where the gel microparticles were suspended into a 0.5% w/w aqueous solution of chitosan. The calcium alginate gel microparticles were often washed with a calcium-free solution to remove excess calcium ions before transferring to the chitosan solution. In the second procedure, the chitosan-alginate membrane was formed when both polymers (chitosan and alginate) were in solution, reacting sodium alginate in an aqueous solution of chitosan and calcium chloride. Chitosan solutions were prepared by the addition of chitosan to distilled water containing hydrochloric acid or sodium acetate buffer at pH 5.0 (Table 1). In both procedures, the microparticles were washed twice with distilled water, collected by filtration, rinsed with ethanol to avoid agglomeration and then finally dried under vacuum for 24 h, at room temperature..

- **Morphology analysis**

The morphology of dried microparticles was observed using JEOL JSM-5200 Scanning Eletron Microscopy., after the samples have been coated with gold.

- **Particle size analysis**

Volumetric size distribution of the microparticles was determined by laser light scattering using a particle analyser Mastersizer 2000 (Malvern Instr.).

- **The amount of water uptake**

The amoung of water uptake of dried microparticles was performed by measuring periodically the weight increase of microparticulate systems, based on a technique adapted from Vam Kamp et al. [4].

Table 1– Identification e descripcion of the studied procedures.

IDENTIFICATION OF THE PROCEDURES	PROCESS CONDITIONS(**)	
	1^o stage	2^o stage
1 $CaCl_2$ 0,1M	Solution of $CaCl_2$ 0.1M	----
1 QUITacNa+$CaCl_2$	Solution of chitosan 0,5% in TacNa with $CaCl_2$ 0.1M	
1 QUIHCl+$CaCl_2$	Solution of chitosan 0.5% em HCl with $CaCl_2$ 0.1M	----
2 QUIHCl	Solution of $CaCl_2$ 0.1M	Solution of chitosan 0.5% with HCl
2 QUITacNa	Solution of $CaCl_2$ 0.1M	Solution of chitosan 0.5% in TacNa

(**) In all the process the atomized solution was 2% sodium alginate. The 1^o e 2^o stages were performed at 20°C, with agitation. TacNa:0.1M buffer sodium acetate, pH 5.0; HCl: hidrochloridric acid was used to dissolve the chitosan at pH 5.0.

Results and discussion

Mean particles diameter ranging from 60 to 553 um was obtained.

When a comparison was made between the surface morphology of calcium alginate and alginate-chitosan microparticles, remarkable roughness and more porous structure was observed in the chitosan-alginate microparticles. Some properties of the microparticles were depend on the method and the procedure conditions of forming the chitosan-alginate complex. When a sodium acetate buffer was used to prepare chitosan solution, the microparticles surface revealed to be highly porous with open channels, probably due to Na^+, an anti-gelling cation.

The same microparticles also presented a strong tendency to swelling, absorbing over 100 times their own weight.

Table 2- Size distribution of particles obtained by diferents procedures.

SAMPLE	MEAN DIAMETER(*)
1 $CaCl_2$ 0,1M	290
1 QUITacNa+$CaCl_2$	428
1 QUIHCl+$CaCl_2$	410
2 QUIHCl	60
2 QUITacNa	553

(*) The mean diameters(um) are expressed as the weighed mean of the volume distribution D[4,3] The procedures description are in Table 1.

Conclusion

The choice of method for producing the chitosan-alginate microparticles determined their properties like size, shape, surface morphology and stability against swelling, indicating that the polyelectrolyte complex membrane formation the resulting microparticles were influenced by the process conditions and the presence of an anti-gelling cation like sodium.

Acknowledgements
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DD-40 BIOINSECTICIDE FORMULATIONS USING MICROENCAPSULATION PROCESS

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Introduction

Every year, insect pests cause between a 15 and 25% loss of agricultural production worldwide. Various strategies have been used to reduce or control this agricultural damage; the principal strategy is the employment of chemical insecticides [1]. The application of these synthetic compounds has resulted in the stabilized or even increase of agricultural yields. However, this strategy has now become one of the most costly aspects of agriculture. The large-scale and indiscriminate use of nonspecific synthetic products, often toxic to mammals, birds, and fish, has resulted in contamination of environment, destruction of non-target organisms, and the development of pest resistance.

Since the 1960s, biological insecticides (bio-insecticides) have been seen as an environmentally friendly, highly desirable alternative to chemical. The microbial insecticide based on preparations of the bacterium *Bacillus thuringiensis* (Bt) are the most widely used representing 95% of the commercial bio-insecticide. They were used for protection of crops, forests and human health and were estimated to represent 1% of the world insecticide market [2].

Bt is a spore-forming bacterium that produces highly specific insecticide crystal proteins, the δ-endotoxins, during the sporulation. At the end of sporulation, the cells lyses and the spores and toxin crystals are liberated. If ingested by susceptible insects (usually the larvae), the crystals are dissolved into the basic pH of the intestine (9.5-10.5) and the δ-endotoxins, which are protoxin molecules, are specifically cleaved by insect gut proteases. The active toxins cause gut epithelium cell lyses and the death of insect larvae.

Because of their high specificity and presumed environmental safety, δ-endotoxins have been successfully used as bio-insecticides against lepidopteran, dipteran and coleopteran pests. Commercial Bt products generally consist of a mixture of spores and crystals, produced in large reactors and applied as foliar sprays, much like synthetic insecticides. However, their performance is often considered to be poorer than that of chemicals insecticides because Bt products act slowly and are not stable in the environment after spraying. They are rapidly inactivated by exposure to sunlight or other environmental factors [3]. Consequently, the duration of pest control is often too short and its use on many crops is not cost-effective because too many applications are required.

The development of an appropriate formulation which are economical and which can deliver a viable organism or an intact protein are critical for successful bio-control products used against plant pathogens. These formulations should provide desirable characteristics for the microbial insecticide, such as long shelf life and appropriate survival in soil as well as sufficient cell density and activity. Furthermore, products should be non-toxic, non-polluting and consistent in quality.

Many different procedures for microencapsulating cells and proteins have been proposed. Use of polysaccharides for gel entrapment has been developed into a challenging method and the use of alginate gel microbeads stands out as the promising and versatile method. This microencapsulation procedure can be carried out under very mild conditions and is therefore compatible with most living cells.

The aim of this work was the development of microparticulated formulations containing encapsulated spores and δ-endotoxins of *Bacillus thuringiensis* var. *kurstaki* (Btk), using alginate and chitosan, two non- toxic and natural polymers, that will protect the microbial bio-insecticide throughout the storage, the reactor mixing and weather conditions after the application.

Experimental methods

- **Materials**

Sodium alginate of medium viscosity (4000cps for a 2% solution at 25°C) is obtained from SKW-Biosystems, named Saltialgine 1100x. Chitosan was of low molecular weight with a viscosity of 10cps, measured at a concentration of 0.5% in HCl, pH 5.0, at 25°C. All others reagents were analytical grade.

- **Bacterial strain**

B.thuringiensis var. *kurstaki* (Btk) was isolated from a Brazilian soil sample and deposited in Microbial Germplasma Bank at EMBRAPA-Genetic

Resources and Biotechnology. The bacterial suspension was obtained by fermentation that was done in IPT-Group of Biotechnology. The fermentation beer was centrifuged in 10,000rpm, at 20°C and washed with saline for obtaining of Btk suspension.

- **Microencapsulation of Btk**

Calcium alginate and Chitosan-alginate microparticles containing spore/toxin are formed by atomizing dispersions of the sodium alginate solution and Btk suspensions into an aqueous solutions of the counter ions 0.1M calcium chloride at 20^{o}C. When chitosan-alginate microparticles were prepared, calcium alginate gel microparticles were first formed by atomizing a 2% w/w solution of sodium alginate mixed with a Btk suspensions into a 0.1M calcium chloride solution at 20^{o}C, followed by a chitosan-alginate membrane forming stage where the gel microparticles were suspended into the 0.5 % w/w aqueous solution of chitosan, pH 5.0. The calcium alginate gel microparticles were often washed with distilled water to remove excess calcium ions before transferring to the chitosan solution. The gelled microparticles were kept under agitation for 60 min. After this reaction time, they were filtrated, washed twice with deionized water, dehydrated with ethanol and then finally dried under vacuum for 24 h, at room temperature.

- **Enumeration of viable spores**

The number of colony forming units (cfu) in Btk suspension before and after the microencapsulation process was done using the spreading technique after incubation in nutritive agar at 30°C, for 15h. The dried microparticles containing Btk were precisely weighed and dissolved in sterile 100 ml 50 mM sodium citrate solution for releasing the spores entrapped within the microparticles and enumeration of viable spores.

- **Morphology analysis**

The morphology of dried microparticles was examined using JEOL JSM-5200 Scanning Electron Microscopy after the samples were coated with gold. The morphology of water dispersion microparticles was observed using an optical microscope, Eclipe E 400POL model, with a Nikon photographic machine.

- **Particle size analysis**

Volumetric size distribution of the microparticles was determined by laser light scattering using a particle analyser Mastersizer 2000.

- **Bioassays**

The larvicidal activity against *Spodoptera frugiperda* larvae were evaluated in the laboratory of EMBRAPA-Genetic Resources and Biotechnology before and after the Btk had been formulated. The larva of Spodoptera frugiperda is a lepidopteron pest of maize.

Results and discussion

The spores counting into filtrate and washing solution showed that around 97% of the spores were incorporated within the microparticles. The number of viable spores in dissolved microparticule formulation demonstrated no loss of cell viability.

With the addition of Btk into microparticles the morphology of them did not change, maintaining a homogeneous particles distribution with a mean diameter of 60 um. The surface morphology showed a membrane of alginate or alginate-chitosan coating the spores of the microbial insecticide.

When dispersed in water the formulations stayed intact without swelling or releasing the spores.

The bioassays results against *S.frugiperda* reveled that the Btk formulated caused the same percentage of mortality than the original Btk suspension.

Conclusions

The encapsulation of suspensions of Btk containing spores and δ-endotoxins in alginate and chitosan-alginate microparticles did not decrease the larvicidal activity of this bio-insecticide against lepidopteron pests. The formulations developed in this study remained unchanged, did not swell, did not release the spores when dispersed in water and can be useful to be applied by aqueous spray as bio-insecticide in agriculture.

Acknowledgements

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DD-41 A NEW VACCINE AGAINST TUBERCULOSIS BASED ON hsp65-LOADED MICROSPHERES IS ABLE TO ELICIT A SPECIFIC TH1 IMMUNE RESPONSE AFTER INTRATRACHEAL ADMINISTRATION

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Introduction

Bacilli Calmette Guérin (BCG), the only currently available vaccine against tuberculosis (TB), has demonstrated variable efficacy in human trials ranging from 80% to no protective effect [1]. In attempt to develop a new and improved TB vaccine, different sub-fractions of *Mycobacterium tuberculosis* have been suggested as vaccine candidates. More recently, proteins secreted from live mycobacteria have attracted more interest [2,3]. Among them, the hsp65, a 65-kDa-heat shock protein, emerged as a good candidate.

Subunit vaccines consisting of a limited number of protein antigens can meet high safety requirements but the poor immunogenicity of these highly purified proteins emphasizes the need for effective adjuvant capable of inducing an optimal immune response [4]. We have previously showed that, after 4 intravenous administrations, the protein hsp65 entrapped in cationic liposomes can give protective immunity similar from live BCG in a mouse model, whereas the protein in Freund's incomplete adjuvant (FIA) has minimal effect [5]. These results reinforce the importance of choosing the correct adjuvant for a subunit vaccine and suggest that the way by which the antigen is recognized, as an endogenous or exogenous antigen, can drive the immune response towards a protective pattern or not. Moreover, the immunization route is other important factor to be considered. Since most infections including TB, is acquired trough the mucosas, it is logical to hypothesize that the stimulation of mucosal immunity by vaccination may be a more effective approach in preventing the initial infection. Thus, new dosage forms and administration routes can simplify vaccination and compliance, and a major step towards a better vaccination coverage would be the induction of single–dose vaccines with the potential of reducing the number of repeated booster administration required for long-term protection. In this way, microspheres derived from biocompatible and biodegradable polyesters of lactic and glycolic acids (PLGA) have been largely proposed as vaccine carriers and adjuvant [6].

Goals

In this study we evaluated the ability of PLGA microspheres to act as a subunit vaccine carrier and to elicit a local and systemic immune response after intratracheal administration in mice.

Methods

1.Obtaining and characterization of hsp65-loaded PLGA microspheres: Microspheres were obtained by double-emulsion solvent evaporation method. Polymer used was PLGA 50:50 (Resomer RG 505, MW 78,000, from Boehringer Ingelheim, Ingelheim, Germany). Particle diameter distribution was evaluated by laser diffractometry in a Cilas 1064 Liquid apparatus (Cilas, France). Entrapment efficiency was evaluated by protein recovery in water after microspheres dissolution in methylene chloride. In vitro release profile of hsp65 from microspheres was followed over 30 days keeping particles under agitation at 37°C and measuring the amount of protein released in PBS. Protein was measured by spectrophotometry at 600nm using the Coomassie protein assay reagent (Pierce, Rockford, USA). The integrity of encapsulated protein was evaluated by electrophoresis and Western blotting after microspheres disruption as described above.

2.Immunization studies: BALB/c mice, 6-8 weeks old, received 50µg of hsp65 in PBS or 5mg of hsp65-loaded microspheres (corresponding to approximately 9 µg of hsp65) by intratracheal route. Unloaded microspheres were used as control. Mice were killed 30 days after immunization. Specific antibody levels were evaluated in serum by ELISA. Cytokine production in supernatant of splenic cells or in lung cells homogenate was measured by ELISA.

Results and discussion

Recombinant hsp65 was incorporated into PLGA microspheres by the double emulsion/solvent evaporation method. Encapsulation efficiency was 70.16%, corresponding to 1.75 μg of protein/mg microspheres.

The ability of microspheres to target antigen to Antigen Presenting Cells (APC) have been attributed to particles smaller than 10μm. This passive targeting is a very interesting strategy to avoid antigen loss and also to deliver antigen into cell cytoplasm, qualifying the APC to stimulate both CD4+ and CD8+ T cells. The cumulative diameter values indicate that 90% of the hsp65 entrapped microspheres populations were smaller than 10.55 μm, 50% were smaller than 6.23 μm, and 10% were smaller than 1.40 μm. These results show that the process for microspheres obtaining is suitable to produce particles that can be taken up by phagocytic cells. Moreover, electrophoresis and Western blotting analysis showed that the protein extracted from microspheres presents a similar band to purified hsp65 and can be recognized by antibodies from serum of immunized mice, suggesting that the encapsulation technique did not affect the antigen integrity. *In vitro* release test showed that 100% of entrapped protein is released in a 30-days period.

BALB/c mice immunized with hsp65 in PBS or entrapped in microspheres presented serum production of antibodies specific to hsp65. Levels of IgG2a were similar between the groups that received the hsp65, 30 days after immunization. However, levels of IgG1, the isotype associated with a no protective Th2 response, are higher in mice that received free hsp65 if compared to hsp65-loaded microspheres immunized mice, in all periods evaluated. Regarding to cellular immune response, spleen cells of mice immunized with hsp65-loaded microspheres, but not with free hsp65, were able to produce higher amounts of IFN-γ, compared to non-stimulated control, when re-stimulated *in vitro* with the protein. In lung cell homogenate, mice immunized with hsp65 in PBS or entrapped into microspheres presented levels significantly higher of IL-12, compared to mice, which received unloaded microspheres mice. Production of IFN-γ by lung cells was similar for all groups. These data suggest that hsp65-loaded microsphere are able to induce both, systemic and local immune response after administration on lung mucous membrane.

Conclusions

Microspheres obtaining technique was suitable to produce particles with desirable features for a vaccine carrier proposal. The encapsulation method did not affect protein integrity and its ability to be recognized by antibodies. Microspheres administration by intratracheal route was able to elicit both humoral and cellular specific immune response.

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DD-42 IMPROVEMENT OF TH1 IMMUNE RESPONSE BY COMBINATION OF TDM AND PLGA MICROSPHERES AS ADJUVANTS FOR A hsp65-ENCODING DNA VACCINE AGAINST TUBERCULOSIS

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Introduction

Traditional dosage forms for drug development and vaccine carriers have failed to deliver the goods in the fight against tuberculosis (TB). Since BCG, the only currently vaccine available against TB, shows a variable protection, the search for new vaccines have been dramatically increased in the last years.

Beyond antigen, the use of adjuvants with immunomodulatory properties could be a key step in a vaccine development due to its ability to drive the immune response trough a specific pattern. The use of cytokines as immunomodulators have turned out right in several experimental and clinical trials but cost, stability and toxicity related to these compounds are still a problem. Thus, the search for new molecules that could stimulate some classes of cytokines constitutes a strategy to find new vaccine adjuvants.

Trehalose dimycolate (TDM), a glycolipid from the external layer of the cell wall of mycobacteria is a potent immunomodulator that increases nonspecific resistance to infectious agents and limits tumor growth [1,2]. To evalulate the role of TDM on immunopathogenesis of TB we developed a model based on TDM-loaded poly-DL-lactide-coglycolide (PLGA) microspheres. This system induced high levels of IL-6, TNF-α, IL-12, IL-10, IFN-γ and IL-4 production by lung cells after intratracheal administration and high production of nitric oxide (NO) in culture supernatants of bronchoalveolar lavage cells [3]. Direct or indirect attraction of macrophages by TDM to the site of inoculation may be one component of its mechanism of action. Other TDM action would be the priming of newly arrived macrophages at the site of inoculation. The ability of macrophage to simultaneously secrete cytokines and NO *in vivo* and *in vitro* after TDM stimulation suggests that TDM could be of particular relevance for the immune response in TB. In addition, the cytokines induced by TDM have great potential as immunomodulators and adjuvants in both infectious diseases and cancer. We propose that cytokine inducers such as TDM may be attractive candidates for therapeutic and also for using as an adjuvant for DNA vaccines or gene therapy against tuberculosis.

Our previous works with a DNA vaccine expressing hsp65 (a 65 kDa heat shock protein from *M. leprae*) showed that, after intramuscular administration of 100 μg on 3 or 4 occasions, at 3-4 week intervals, the immune response profile was predominantly of TH1 type (abundant IFN-γ, minimal IL-4) [4]. It was also possible to observe that a new system based on the entrapment of the plasmid into PLGA microspheres is able to reduce the amount and number of administrations of DNA required to stimulate the immune response. We are now exploring the improvemento of the immune response by associating aN immunomodulator to our system, combining the adjuvant mechanisms of TDM and microspheres to deliver a DNA vaccine against TB.

Goals

In this study we evaluated the immune response in mice immunized with pCDNA4-hsp65 plus TDM-loaded PLGA microspheres before and after a challenge infection with *M. tuberculosis*.

Methods

- **Obtaining of DNA plus TDM-loaded PLGA microspheres**

Microspheres were obtained by double-emulsion solvent evaporation method. We used a plasmid containing the gene encoding the hsp65 protein from *M. leprae* (pCDNA4-hsp65) or only the vector pCDNA4 as control. TDM was introduced in the organic phase containig the polymer. Polymer used was PLGA 50:50 (Resomer RG 505, MW 78,000, from Boehringer Ingelheim, Ingelheim, Germany).

- **Immunization studies**

Young adult BALB/c mice, 6-8 weeks old, received a single dose of 5mg of microspheres containing pCDNA4-hsp65 and TDM by intramuscular route. Unloaded, TDM-loaded or pCDNA4 plus TDM-loaded microspheres were used as control. Mice were challenged with 10^5 CFU of *M. tuberculosis* H37Rv, 30 days after immunization and killed 60 days after infection. Specific antibodies levels were evaluated in serum before and after challenge using ELISA technique. Levels of cytokines produced in lung cells homogenate of challenged mice were measured by ELISA.

Results and discussion

The combination of TDM and PLGA microspheres as DNA vaccine adjuvants which result in three main mechanism of adjuvanticity such as carrying, targeting for phagocytic cells and immunomodulation, presented exciting results. It is important to emphasize that the dose of TDM used in this study is 10 times lower than that in the microspheres used to evaluate the role of TDM in the patogenesis of TB as described elsewhere [3].

BALB/c mice immunized with pCDNA4-hsp65-TDM-loaded microspheres presented serum production of antibodies specific to hsp65. Levels of IgG2a were higher than the controls while levels of IgG1 were not significantly different from vector plus TDM-loaded micrspheres immunized mice. These data suggest that pCDNA4-hsp65 plus TDM-loaded microspheres could elicit a protective immune response since the IgG2a class of antibody is related to a TH1 immune response, which is important in TB control. After challenge, levels of IgG1 specific for hsp65 increase probably due to immunomodulatory effects from other mycobacterial compounds at antigen recognition site. However, the level of IgG2a remains high. Production of IFN-γ, IL-12 and IL-10 in homogenate of lung cells from challenged mice was higher in mice immunized with pCDNA4-hsp65-TDM-loaded microspheres than the other groups. These results could suggest that the mice immunized with pCDNA4-hsp65 plus TDM-loaded microspheres may have greater ability to control the infection than the other groups. In order to confirm this hypothesis, we are evaluating now the number of colony forming unit (CFU) in lungs of immunized and challenged mice with a virulent strain of *M. tuberculosis*.

Conclusions

The co-entrapment of TDM and pCDNA4-hsp65 into PLGA microspheres seems to be an interesting tool to elicit protective immune response against tuberculosis in a mouse model.

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DD-43 EFFECT OF DIFFERENT CYCLODEXTRINS ON THE INSULIN-LOADED PLGA MICROSPHERES FORMULATIONS

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Introduction

The treatment of diabetic patients with insulin requires the daily parenteral administration of this hormone to control the metabolic processes in the organism. Many problems are associated to this therapy where, the daily injections can result in undesirable reactions such as the peripheral hyperinsulinemic effects.

New strategies for drug administration and also alternatives routes are the main aims of many groups form academy and industries. One of these alternatives that is receiving special interest concerns the use of biodegradable microspheres in which insulin can be entrapped and delivered in a controlled way. Indeed, this formulation protects the hormone improving its stability. This system have the potential to solve many problems associated with the commonly use of injectable therapy and nowadays other alternatives routes such as oral and pulmonary are in trials.

One of the main problems related to the encapsulation of the insulin concerns its solubility since a small volume of the solution containing drug is entrapped if we consider the double-emulsion method for preparation. In attempt to increase the ratio drug/polymer (%w/w) in the microspheres we have considered the improvement of solubility of this hormone by means of cyclodextrins complexes. Beside these properties cyclodextrins are also able to promote the drug absorption and stability of complexed drugs.

Goals

The aim of this work was the evaluation of two selected cyclodextrins to complex the hormone insulin in aqueous solutions. These solutions were used to prepare the PLGA microspheres and we evaluated the physicochemical characteristics of the systems.

Methods

- **Materials**

Porcine insulin (INS) (28.1U/mg), Poly (DL-lactide-co-glycolide) 50:50 (Resomer® RG 505, MW 70800), 2,6-dimethyl-β-cyclodextrin (DMCD) (Sigma Chemical Co), Hydroxipropyl-β-cyclodextrin (HPCD) (C* Cavitron^{TM} 82006), polyvinyl alcohol (PVA) (Mowiol 40-88, MW 127000).

- **Preparation of insulin/cyclodextrin (INS:CD) complexes**

The complex INS:CD was prepared by dispersion of these substances in PBS, pH 7,4. For HPDC the molar ratios were 1:1, 1:2, 1:5 and 1:10. The concentration of insulin was closed to the saturation point. For DMCD, 12.5mg of insulin was weighed and dispersed in 125µl of PBS, in the presence of DMCD in the molar ratios of 1:2, 1:3 and 1:5. In all the cases the solutions were maintained under magnetic stirring at room temperature for 4h.

- **Preparation of microspheres**

PLGA 50:50 microspheres were prepared by double-emulsion method followed by organic solvent evaporation. 125µl of insulin:cyclodextrin solution was dispersed in 30ml of methylene chloride containing 200mg of PLGA 50:50. This primary emulsion was kept under strong agitation (13,500 rpm) for 1 minute using a Ultraturrax T25 (Ika - Germany). This primary emulsion was added at 100mL of PVA (5%) to obtain the multiple emulsion W/O/W. The system was kept under agitation at 900rpm at room temperature to allow solvent evaporation. The microspheres were centrifuged, washed three times with distilled water, freeze-dried and stored at 4 °C.

- **Determination of the average size**

Microspheres were dispersed in sterile water and the diameter of the microparticles was evaluated by laser difratometry using a CILAS granulometer 1064L (CILAS - France).

- **Determination of insulin loading**

10mg of microspheres were accurate weighed and solubilized in acetonitrile. The insulin precipitated was centrifuged at 39100 x g for 5 minutes and the pellet was dissolved in 1mL of HCl 0.1M. The insulin was finally assayed by HPLC (Waters Corporation, USA).

- **Kinetic release**

Drug release profile was carried out at 37°C in 0.05M Tris isotonic buffer, pH 7.4 and also in simulated gastric/enteric medium without enzymes. The insulin was recovered as described above in different time intervals and assayed by HPLC.

Results and discussion

The effect of cyclodextrins on the average size of microspheres was more evident when the drug was associated with HPCD. As seem in Table I, the average size varied from 2μm when free drug was used to almost 4μm when HPCD was used in the proportion of 1:10. This effect was not observed when DMCD was used in the formulations.

Table 1
Influence of cyclodextrins on the average size of microspheres.

Molar ratio	Average size (μm)	
INS:CD	HPCD	DMCD
1:0	2.06	2.06
1:1	3.04	-
1:2	3.37	2.14
1:3	-	2.35
1:5	3.78	2.53
1:10	3.84	-

The complex INS:HPCD showed to improve the drug loading. When free drug was used 0.42% w/w was entrapped into microspheres. When associating to HPCD this value increased almost six times (2.29%). It might be related to the higher solubility of insulin in the presence of HPCD. Similar results, although less significant, was observe when DMCD was used. The drug loading varied from 0.42% to 1.85% when DMCD was used in the molar proportion of 1:5 (Table II). In both situations the selected molar ratio was 1:5.

The drug release study revealed that the complexes of insulin with both cyclodextrines reduced the burst effect if compared when insulin is used alone in the inner aqueous phase. For simulated gastric medium, the release revealed a biphasic profile showing a faster release in the acid pH.

Table 2
Insulin loading into PLGA microspheres using the complex INS:CD.

Molar ratio	% (w/w) of insulin	
INS:CD	HPCD	DMCD
1:0	0.42	0.42
1:1	0.56	-
1:2	2.24	0.31
1:3	-	1.53
1:5	2.29	1.85
1:10	1.52	-

Conclusion

This study demonstrated that the complex INS:HPCD provides an increase on the average diameter and also improves drug loading into microspheres. Similar results had been observed when using DMCD. This association can contribute to increase the drug stability and also to improve drug absorption since cyclodextrins have been described as an absorption promoter. Both formulations showed to be able to reduce the glicemia after intratracheal administration in mice and rats as described elsewhere.

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DD-44 NANOENCAPSULATION REDUCES QT-INTERVAL PROLONGATION OF ECG PROVOKED BY HALOFANTRINE IN A RAT MODEL

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Introduction

Severe malaria is considered a medical emergence, and hence the development of an intravenous formulation of a very active and fast-acting antimalarial drug against chloroquine-resistant strains of *Plasmodium falciparum* is a priority [1]. Halofantrine (Hf) is an appropriate candidate and well tolerated drug to treat malaria. However, it often exhibits low bioavailability by the oral route as its absorption can be kinetically limited by low rates of dissolution and capacity limited by poor solubility. The intravenous nanocapsules formulation of Hf (Hf-NC), developed in previous work [2], presents a better pharmacokinetic profile when compared with a solubilized form of halofantrine in PEG/dimethylacetamide/glucose injected intravenously [3]. The area measurement from the plasma concentration curve of Hf was increased 6 times with Hf-NC when compared with the solubilized form. Furthermore, we have also observed a marked reduction of toxicity (DL_{100}) of Hf-NC in a mouse model of malaria.

The large use of Hf is limited by its propensity to prolong the QT-interval of the electrocardiogram (ECG). QT-interval is a widely used measure of the ventricular repolarization process and its prolongation may be associated with a risk of sudden death [4].

The aim of the present work was to verify the effects of different formulations (nanocapsules or solution) of Hf in the ECG QT-intervals in anaesthetized rats at doses as higher as 150 mg/kg/day injected intravenously.

Experimental methods

- **Materials**

Halofantrine chlorhydrate was a gift from SKB (UK). Poly(ε-caprolactone) PCL, Mw 42.5 kDa was provided by Sigma-Aldrich (USA). Epikuron 170 (70 % soy phosphatidylcholine) was purchased from Lucas Meyer (France). Miglyol 810N was kindly provided by Hülls (Germany). The solvents were analytical grade and all other chemicals were commercially available.

- **Nanocapsules preparation**

The free base was prepared by treating the solution of Hf salts as previously described [5]. Nanocapsules were prepared with 6mg/ml of the polymer (PCL) which were solubilized in acetone solution with 75 mg of Epikuron 170 and Miglyol 810N containing Hf as free base form (10-15 mg/ml). This organic solution was poured into the external aqueous phase containing Poloxamer 188 before evaporation of the acetone under vacuum. Hf base content was analysed by HPLC [6].

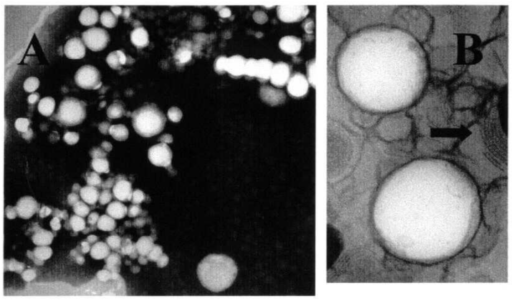

Figure 1. Transmission Electron Microscopy of Halofantrine Nanocapsules at 1 mg/ml of NC suspension (A). Detail of NC wall and internal oily core (B).

- **Determination of the toxic dose in rats**

Male Wistar rats received different doses of unloaded NC, Hf-loaded NC, Hf solution and adjuvants of Hf vehicle. The doses of Hf used were 100 mg, 150 mg and 200 mg/kg/dose. The effects observed in order to estimate toxicity, were grooming, ataxia, alterations of breathing frequency, trembling, arrhythmia, alterations in the postural and locomotion control, convulsions and death.

- ***In vivo* studies**

Male Wistar rats weighting 200-300 g were divided in three groups that receive unloaded PCL nanocapsules, Hf solution (10 mg Hf.HCl/ml PEG/DMA) and halofantrine PCL nanocapsules (10-15mg Hf/ml) at 150 mg/kg/dose. The animals were anaesthetized with sodium thiopental intraperitonially (70 mg/kg). The femoral artery and vein were catheterized for arterial pressure (AP) measurement and drug injection, respectively.

The AP and ECG signals were conditioned by an electrocardiographic amplifier and analog-to-digital converted with a sampling frequency of 1200 Hz and a resolution of 12 bits. Records of 10 and 30 minutes were taken before and after injection of the different formulations, respectively.

Results

The NC obtained were stable and their mean size was below 200 nm. The drug loading was higher than 99.0 %. The preparations were filtered in 0.22 µ sterile filter before *i.v.* injection. Table 1 shows the toxic effects observed in rats after *i.v.* injection of the different formulations during experiments of maximal tolerated dose determination.

Table 1. Toxic effects of different Halofantrine formulations in rats after *i.v.* injection.

Dose (mg/kg)	**Fast injection**	**Slow Injection**
NC		
100	1,2(+--)	nd
150	1,2(+- -)	nd
200	1,2(+--)	nd
Hf-NC		
100	nd	1(+--),3(++ -)
150	nd	1(+--),4(+- -)
200	1(+--), 2(++-),3(+- -)	nd
Hf..HCl		
100	1(++-), 2(++-),3(+- -)	1(++-), 2(++-),3(+- -)
150	1(+++), 2(++-),3(+--)	1(+++), 2(++-),3(+- -)
200	4(+++), 5(+++), ✝	nd
Vehicle		
100	1(+--), 2(+--), 4(+- -)	nd
150	1(+--), 2(+--), 4(+- -)	nd
200	1(+--), 2(+--), 4(+- -)	nd

1-grooming; 2-trembling/icthing; 3-respiratory frequency; 4-ataxia; 5-convulsion; ✝ death, nd = not determined.

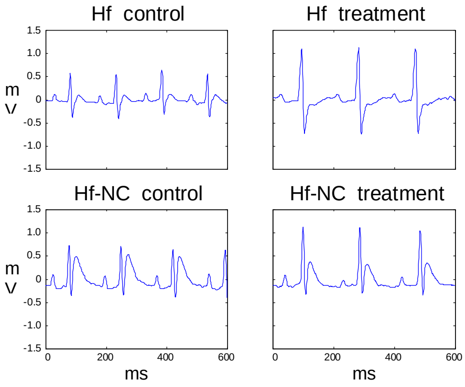

Figure 2. ECG of rats that received an *i.v.* injection of Hf solution and Hf-NC, before (control) and after treatment.

No significant alterations of arterial pressure (AP) were observed after injections of all formulations, even with Hf solution and with Hf-NC. Hf solution induces a significant increase of 16 % in QT-interval prolongation just after injection. However, after the *i.v.* injection of NC formulation, the increase was only 3 % (an example is showed in figure 2). Unloaded NC didn't induce a significant increase in QT interval.

Conclusion

The results suggest that encapsulation of Hf is able to reduce QT-interval alterations provoked by this drug when compared with non-capsulated solution. Hf seems to be inside nanocapsules after *i.v.* injection, which cause significant cardiac protection, reducing consequently the cardiotoxicity. Further studies of relationship between doses and pharmacological cardiac effects, time-dependence, and morphological analysis of the ECG are in progress.

Acknowledgements

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DD-45 DICLOFENAC-LOADED SPRAY-DRIED POLYMERIC NANOPARTICLES PREPARED WITH MINIMAL SURFACTANT CONCENTRATION

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Introduction

Recently, we have described the use of spray-drying technique for drying polymeric colloidal systems [1]. Nanoparticle suspensions prepared with diclofenac or indomethacin as drug, poly(ε-caprolactone) as polymer, Miglyol 810 as oil phase (only for nanocapsules), lipophilic and hydrophilic surfactants were spray-dried in the presence of a silicon dioxide micro-powder (drying adjuvant). The resulting powders were easily redispersed in water. The scanning electron microscopy analyses of the powders showed the silicon dioxide microparticles completely covered by nanostructures of about 300 nm for nanocapsules formulations and 70 nm for nanospheres formulations [2,3].

The aim of the present study is to investigate the influence of the surfactant concentration on the physico-chemical stability of spray-dried powders containing diclofenac.

Materials and Methods

Diclofenac sodium was obtained from Sigma (St. Louis, USA), polysorbate 80 from Delaware (Porto Alegre, Brazil). Epikuron 170 , a phospholipid mixture, was supplied by Lucas Meyer (Hamburg, Germany) and Miglyol 810 , a caprilic/capric triglyceride, was obtained from Hulls (Puteaux, France). Poly(ε-caprolactone) (60,000), PCL, was supplied by Aldrich (Strasbourg, France). Aerosil 200 , colloidal silicon dioxide, was obtained from Degussa (Germany). All other chemicals and solvents used were of pharmaceutical grade. All reagents were used as received.

- **Preparation of the diclofenac free acid**

An aqueous solution of sodium diclofenac was acidified to pH 2.0 with 5 M HCl. The aqueous phase was extracted with chloroform and the organic phase treated with anhydrous $NaSO_4$, filtered and evaporated. The diclofenac free acid was recrystallized from ethanol-water 1:1 (v/v).

- **Preparation of the polymeric colloidal suspensions**

Submicromic suspensions were prepared by nanodispersion [4]. The organic solution consisted of diclofenac free acid (1.0 mg/mL), PCL (10.0 mg/mL), Miglyol 810 (0.03 mL/mL), Epikuron 170 (Table 1) and acetone. The organic solution was added under moderate magnetic stirring to the aqueous solution of polysorbate 80 (Table 1). The suspension was concentrated under reduced pressure for acetone elimination and the drug concentration was adjusted to 1 mg/mL with distilled water. The particle size was measured by laser light scattering (Nanosizer , Coultronics, Andilly, France).

Table 1. Nanoparticle formulations.

	Particle size (nm ± SD)	Epikuron 170 (mg/mL)	Polysorbate 80 (mg /mL)
A	181 ± 28	7.66	7.66
B	181 ± 51	6.13	6.13
C	177 ± 24	4.60	4.60
D	186 ± 51	3.06	3.06
E	225 ± 55	1.53	1.53
F	179 ± 49	7.66	-
G	292 ± 90	-	7.66

- **Preparation of the spray-dried powders [2]**

To the suspensions were added 3.1 % (w/v) of Aerosil 200 and the mixtures were fed into a mini-spray-dryer Büchi MSD 190 (Flawil, Switzerland), provide with a 0.7 mm pneumatic nuzzle, in tangential flow at a feed speed of 3 mL/min. The inlet temperature was 150 ± 4 °C; the outlet temperature was 110 ± 4 °C, and the exhaustion was fixed at level 14.

- **Assay procedure**

The suspensions were treated with acetonitrile for total DIC determinations. The spray-dried products were dispersed in acetonitrile under magnetic stirring for 60 minutes and filtered through a hydrophilic membrane (0.22 μm – HWGP, Millipore). The recovery of diclofenac from the spray-dried powders was estimated by correlation of theoretical and practical concentrations. DIC was assayed by HPLC [5] after 0, 3 and 6 months of storage at room temperature. The system consisted of Shimadzu (Kyoto, Japan) auto-sampler CIL-10A, a Shimadzu pump LC-10AD, a Shimadzu UV-detector SPD-10A and a Waters Nova-Pack C18 (3,9 x 300 mm) column. The mobile phase consisted of acetonitrile/water (65:35 v/v) adjusted to pH 5.0 with 10 % acetic acid. DIC was detected at 280 nm with a retention time of about 3.4 min.

- **Scanning electron microscopy (SEM)**

After 6 month of storage, the spray-dried powders were examined by SEM (Jeol Scanning Microscope, JSM-5800). Samples were analyzed after they have been gold sputtered.

Results and Discussion

The suspension prepared without surfactants (entry **H**, Table 1) presented considerable macroscopic precipitation before the evaporation of the solvent (acetone). Diclofenac content in nanocapsule suspensions (**A**-**F**) remained constant after 6 months of storage at room temperature (Table 2). In contrast after 6 months of storage, considerable decrease of DIC content was observed for the suspensions prepared without Epikuron 170 .

Table 2. Nanocapsule suspensions.

	Drug content (mg/mL) ± SD		
	0 months	3 months	6 months
A	1.06 ± 0.02	1.02 ± 0.04	1.08 ± 0.04
B	1.11 ± 0.09	1.07 ± 0.03	1.02 ± 0.02
C	1.07 ± 0.01	1.02 ± 0.04	1.08 ± 0.04
D	1.03 ± 0.04	1.01 ± 0.02	1.05 ± 0.03
E	1.00 ± 0.01	0.89 ± 0.04	0.94 ± 0.08
F	1.07 ± 0.10	1.02 ± 0.05	1.05 ± 0.01
G	0.96 ± 0.01	0.70 ± 0.16	0.61 ± 0.10

After spray-dried, the corresponding powders of suspensions **A**-**G** were obtained with 60 to 70 % of yield. After 6 months of storage at room temperature, all powders presented drug recovery between 90 % and 100 % (Figure 1), excepting powder **G** (without Epikuron 170) which showed a significant decline of DIC content (83.5 %). After 6 months, for all formulations SEM analyses showed nanostructures about 200 nm covering the microparticles of silicon dioxide, as showed in the Figure 2 for formulations **E** and **F**.

Conclusion

Excepting the formulation prepared without both surfactants, all spray-dried powders obtained from NC suspensions prepared with different concentrations of surfactants had similar morphological characteristics and showed the presence of nanostructures with homogeneous distributions onto the surface of drying adjuvant. However, the minimal concentration of each surfactant compatible with the powders stability was 1.53 mg/ mL. The formulation prepared using only the lipophilic surfactant at 7.66 mg/mL was also stable after 6 months of storage. Work is in progress to evaluate the minimal concentration of the lipophilic surfactant in order to obtain a satisfactory formula.

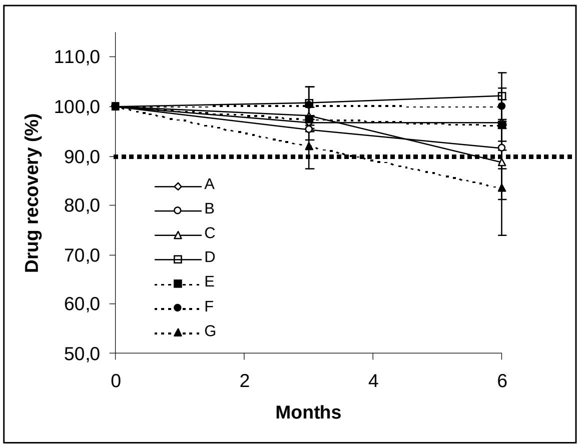

Figure 1. Drug recovery of spray-dried powders **A**-**G**.

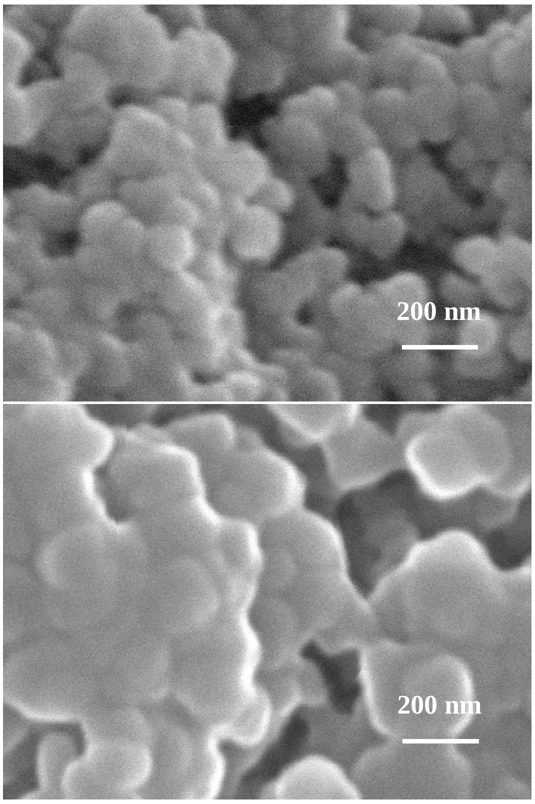

Figure 2. SEM micrographs of formulations **E** (upper) and **F** (bottom).

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DD-46 CYTOTOXICITY AND ANTITUMORAL ACTIVITY OF PLGA-NANOCAPSULES CONTAINING USNIC ACID

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Introduction

The usnic acid, a low-molecular-weight lichen constituent, has extensively been studied mainly because it presents substantial antimitotic activity. Its antibacterial and antimycotic properties, as well as activity against human neoplasic cell lines are well-known [1]. However, applications of usnic acid in anticancer therapy have been rather limited due to its high *in vivo* toxicity [2].

Alternative nanoparticulated carriers have been developed aiming to decrease toxic effects of anticancer compounds towards normal tissues and to increase their efficiency against tumors [3].

The goal of the present work is both to evaluate the cytotoxicity and the antitumor activity of free and encapsulated usnic acid into PLGA-nanoparticles.

Experimental methods

• Materials

Usnic acid extracted from *Cladonia substellata* (Paraiba, Brazil), Poly (lactic-co-glicolic acid) PLGA (50:50); Epikuron 200®, Synperonic F68®, soya oil, tween 80, 3,4–dimethylthiazol–2–YL-2,5-diphenyltetra- zolium bromide (MTT), L-glutamine and trypsin.

• Preparation of PLGA-nanocapsules

PLGA-nanocapsules containing usnic acid were prepared by the interfacial deposition of a preformed polymer method [4].

• *In vitro* cytotoxicity

Cytotoxicity of free and encapsulated usnic acid was analyzed on NCI-H 292 cells cultured in MEM (Minimum Essential Medium). The cell viability was tested by the exclusion of Trypan blue dye. Cell suspensions were then diluted to 10^5 cells. ml^{-1} and aliquots of 220 µl of such suspensions were seeded on 96-well plates. After 24 hours, samples of usnic acid solution or PLGA-nanocapsules containing usnic acid, at concentrations ranging from 2.5 to 20µg ml^{-1}, were tested. DMSO or MEM at the same concentrations was used as a control. The cytotoxic effects of usnic acid were evaluated by the MTT colorimetric method after 72 hours of incubation. Cell morphological abnormalities were observed by microscopy using the Giemsa technique

• Antitumor activity

Antitumoral activity was verified against solid tumor sarcoma 180 in Swiss mice (25-30g). Sarcoma 180 grown in the ascites of mice in an appropriate time scheduled was diluted with saline in order to obtain 5. 10^7 cells/ml suspension. Then 0.2 ml of the suspension was injected s.c. into the posterior flank of 5-week-old male mice. Chemotherapy was started 24 hours after implantation and maintained for 7 days, using an $LD_{50}/10$ concentration. After this time, mice were sacrificed and tumor, liver and kidneys were removed after bleeding. Organs were washed with saline, weighted, fixed with 10% formaline, and submitted to histological studies. Antitumoral activity was estimated by the tumor inhibition [5].

Results and discussion

The viability of cells treated at different concentrations of free and encapsulated usnic acid is shown in Figure 1. Concentrations required to inhibit 50% of cell growth (IC_{50}) were 14 and 20 µg.ml^{-1}, for free and encapsulated usnic acid, respectively. The effect of the usnic acid treatment on the cell morphology is displayed in Figure 2. At 10 µg.ml^{-1} concentration of free usnic acid, abnormalities were detected in cellular adhesion and cellular clusters were formed. The reduction of cell adhesion can be observed by the presence of empty spots on the cell monolayer as shown in figure 2b. Irregular cytoplasm, extensive areas of vacuoles containing basophilic material and naked nuclei were also observed. However, at low concentrations varying from 2.5 to 5.0 µg. ml^{-1}, cellular morphological alterations were less marked. The cells treated with encapsulated usnic acid formed a monolayer almost similar to that of the control. However, a cytoplasmic vacuolization and a condensed appearance of mitotic cells (Figure 2c) can be identified.

Results of antitumor activity demonstrated encapsulation of usnic acid into PLGA-nanocapsules promoted a 66% increase of tumor inhibition as compared with free usnic acid treatment (Figure 3).

Animals treated with encapsulated usnic acid presented an average weight loss of only 5%. In contrast, those submitted to free usnic acid treatment presented a 26% body weight reduction.

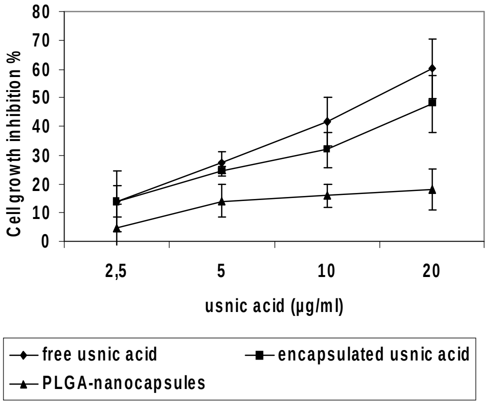

Figure 1. Effect of PLGA-nanocapsules containing usnic acid on NCI -H 292 cell viability.

Histological analysis of treated tumors showed extensive necrotic areas. Morphological alterations were detected in the liver epithelium for both free and encapsulated usnic acid treatment (Figure 4). Vacuolization of hepatocytes and a mild lymphocyte infiltration in portal spaces can be seen in animals treated with free usnic acid. In contrast, no histological changes were noticed in the treated animals' kidneys.

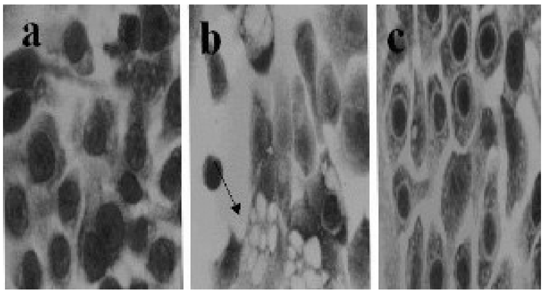

Figure 2. Morphological aspects of NCI H-292 cells (100 X): a) control; b) free usnic acid; c) encapsulated usnic acid treatment.

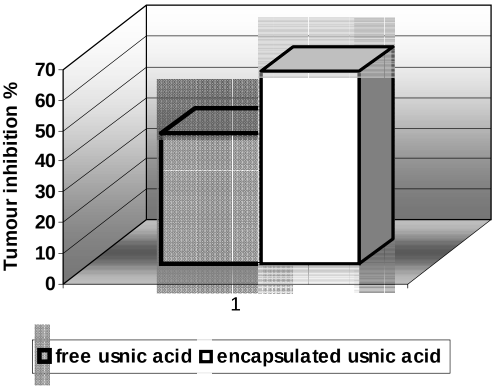

Figure 3. Antitumoral activity of free and encapsulated usnic acid against sarcoma -180.

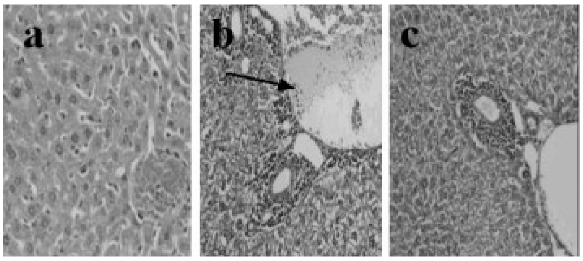

Figure 4. Histological analysis of liver in treated animals (H&E-50X): a) control; b) free usnic acid; c) encapsulated usnic acid.

Conclusions

This work showed that encapsulation of usnic acid into PLGA-nanocapsules reduces *in vitro* cytotoxicity and improves its antitumoral activity. Current studies are being carried out in our laboratory to investigate *in vivo* toxicity of PLGA-nanocapsules containing usnic acid. Preliminary results suggest encapsulation can be a potential alternative to allow the use of usnic acid in cancer therapy.

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DD-47 BIODEGRADABLE NANOPARTICLES LOADED WITH *Bauhinia monandra* LECTIN

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Introduction

Lectins are carbohydrate-binding proteins widely used in biochemical, immunochemical, and histochemical studies. They are recognition proteins of ubiquitous distribution in nature, which are involved, in numerous cellular processes through their characteristic structure and interaction mechanisms [1]. These characteristics have attracted the interest of pharmaceutical scientists for oral delivery, due to their good resistance to acidic pH and to the presence of binding sites along the gastrointestinal tract.

The genus *Bauhinia* (Febaceae) contains a number of ornamental species, which are well distributed in Brazilian cities; they have been used as forage, as human food and in popular medicine for the treatment of diabetes and as a diuretic. As it is the case with lectins, BmoLL showed a primary monosaccharide specificity, however, it did not recognize glycoproteins. [1]

An extensive research work was carried on to develop nanoparticulate systems able to encapsulate water-soluble compounds, such as proteins and vaccines. Protein-loaded poly (lactide-co-glycolide) (PLGA) nanospheres were developed and characterized [2,3]. Stealth poly (lactide)(PLA) - polyethylene glycol (PEG) nanoparticles were proposed as protein carriers for nasal administration [4]. Human serum albumin was encapsulated within PEG-coated nanospheres [5].

However, all the preparation methods, based on a double emulsion, procedure involve the use of organic solvents and sonication steps, which can be deleterious for the biological activity of the entrapped molecules. The aim of this study was to optimize the entrapment of BmoLL lectin in biodegradable nanoparticles and to prove its usefulness as a targeting carrier.

The effects of various parameters involved in the fabrication of biodegradable nanospheres were evaluated with regard to the hemagglutinating activity of the lectin of Bauhinia monandra (BmoLL).

Experimental Methods

- **Hemagglutination assay**

The evaluation of the hemagglutinating activities (HA) was performed with glutaraldehyde-treated rabbit erythrocytes [6].

- **Effect of the exposition to ultrasounds and to Ultra-Turrax on the activity of BmoLL lectin**

250 µl of protein solution of lectin BmoLL – 41 µg/ml (citrate phosphate buffer, pH 6,5)- were added to 2 ml of distilled water and sonicated or agitated for 30 to 60 seconds.

- **Effect of organic solvent on BmoLL lectin**

100 µl of an solution of lectin BmoLL BmoLL – 41 µg/ml (citrate phosphate buffer, pH 6,5)-, 2ml of organic solvent (methylene chloride or acetone), and 4ml de distilled water were mixed together. The organic solvent was evaporated by stirring.

- **Preparation of the nanospheres**

Nanospheres were prepared by a double emulsion method [3], using different polymers (PLA, PLGA and polycaprolactone-PCL) and sodium cholate (SC) or polyvinyl alcohol (PVA).

- **Nanospheres characterization**

The size distribution was determined in distilled water at 20°C using a N4 Plus Coulter. The amount of BmoLL lectin entrapped in the nanoparticles was determined by an assay of the supernatant after two ultra centrifugations (25000rpm / 15 minutes and 40,000 rpm/30minutes), using the Lowry - Peterson method [7].

Results and Discussion

No difference was observed in the haemagglutinating activity of the BmoLL lectin after three sonication steps of 30 and 60 sec, or after ultra-turrax. Moreover, mixing aqueous solutions of lectin with organic solvents such as dichloromethane or acetone, followed by solvent evaporation, didn't affect the haemagglutinating activity, showing the good stability of this lectin under the various experimental conditions used for the manufacture of the nanospheres.

We optimized the parameters involved in the preparation of the nanospheres (ratio organic to aqueous phase, polymer concentration, type of surfactant) in order to obtain monodisperse particles with a diameter lower than 200 nm. The diameter of the nanospheres prepared using SC was three times lower than the one obtained using PVA as a surfactant.

Finally, nanospheres with a main diameter around 150 nm were obtained whatever the nature or the molecular weight of the polymer used (PCL 2000g/mole, PLGA 50/50 21000g/mole, PLGA 75/25 26000g/mole and PLA 42000g/mole) [Table 1].

The highest rate of encapsulation of the lectin BmoLL was obtained by using the copolymer PLGA 50/50 (68.5% ± 5%), whereas the nanospheres prepared with PCL 2000 g/mole presented the lowest encapsulation rate (40%±8%) [Table 1].

Thus, loading of about 1.5µg of protein/mg polymers were obtained. Moreover, the hemagglutinating activity of BmoLL lectin was maintained.

Table 1

Effect of the type of polymer on the nanospheres preparation and on the encapsulation of the of lectin BmoLL.

Polymer	**Mean Diameter (nm±SD)**	**Polydispersity Index**	**Encapsulation (%)**
PCL (2000)	143±47	0.22	40±8
PLGA50/50	155±49	0.19	69±5
PLGA75/25	163±47	0.14	58±8
PLA50(42000)	153±51	0.09	58±5

Conclusions

This study shows that the BmoLL lectin could be efficiently encapsulated into nanospheres of about 150 nm and that the process of preparation of the nanospheres didn't modify the haemagglutinating activity of the BmoLL lectin.

Studies are underway to determine the release profile of the lectin in various media and to investigate the interaction of these particles with Caco-2 cells in culture.

Acknowledgments

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DD-48 APPLICATION OF DIFFERENTIAL SCANNING CALORIMETRY ON THE ASSESSMENT OF INTERACTIONS BETWEEN DRUGS AND MIXTURES OF POLYETHYLENE GLYCOLS AND GLYCERIDES

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Introduction

Over the last decades, intense research has been carried out on solid dispersions [1, 2]. These have been shown to increase the dissolution rates of poorly soluble drugs by increasing solubility and reducing particle size (increasing surface area). Mixtures of polyglycolised glycerides with esters of polyethylene glycol (PEGs) can be used as carriers in solid dispersions of poorly soluble drugs. In this work we use Gelucire® 44/14 [3] as the drug carrier. The first number refers to its approximate melting point and the second one to its hydrophilic-lipophilic balance value, HLB (a value of 14 reflects a water dispersible material). This work focus its attention on solid dispersions with two drugs from the same family but different physical and chemical properties and their characterisation using Differential Scanning Calorimetry (DSC).

Materials and methods

- **Materials**

A mixture of glycerides and polyethylene glycols (Gelucire® 44/14, Gatefossé, France), Propranolol HCl and Pindolol (supplied by Capsifar, Portugal) were used as received.

- **Methods**

For each drug, Propranolol (PRO) and Pindolol (PIN), ten different dispersions with Gelucire (GEL) were prepared. The GEL was melted in a water bath at 75ºC, then the drug was added and dispersed under stirring for 20 minutes. The dispersion thus produced was left to cool down to room temperature. A sample of the dispersion was collected to a DSC standard aluminium crucible (ME 27331). The crucible pan was sealed with a pierced lid and stored for 24 hours for stabilisation, before carrying out the DSC run in a Mettler Toledo Calorimeter (DSC25 and TC11/TA processor, Switzerland). The temperature interval considered was 20ºC up to 200ºC (GEL:PRO dispersions) and 20ºC up to 220ºC (GEL:PIN dispersions) due to the higher melting point of PIN. In both cases the heating rate was 5ºC/min. For each run, a thermogram was produced (Figures 1 and 2) and the correspondent data, endotherms (peaks) and enthalpies, analysed (Table 1). Three thermograms of each raw material were produced and the average of their data were used as references.

Results and Discussion

The thermograms for the two drugs show a distinct behaviour. Figures 1 and 2 illustrate four of the ten thermograms for each drug.

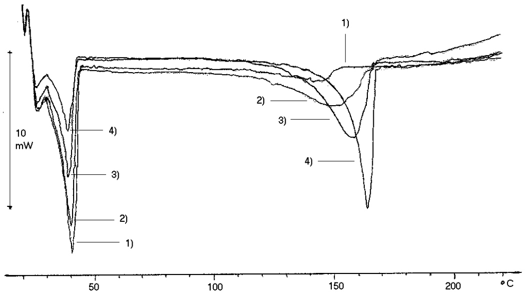

Figure 1 - Thermograms for the binary system GEL : PIN 1) 83 : 17%, 2) 65 : 35%, 3) 50 : 50%, 4) 40 : 60%

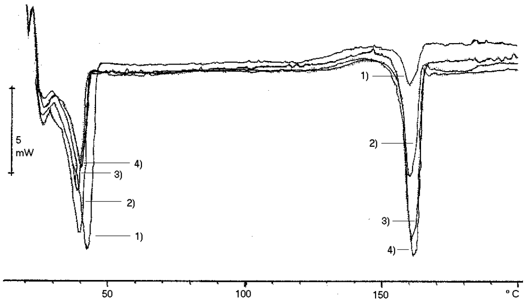

Figure 2 - Thermograms for the binary system GEL : PRO 1) 83 : 17%, 2) 65 : 35%, 3) 50 : 50%, 4) 40 : 60%

For the GEL:PIN dispersions, the drug's endotherm shows a shift for the lower drug concentrations and a broader band. As the drug load increases, the endotherm gets sharper and tends to the PIN endotherm while the GEL endotherm gets smaller but doesn't show significant temperature shifts. In contrast, the carrier endotherm in the GEL:PRO dispersion is more affected by the interaction with the drug whilst the drug endotherm is well defined and rests approximately in the same range of temperatures. This suggests significant differences in the interaction mechanism for the two systems: in the GEL:PRO dispersions, the drug incorporation in the matrix tends relatively fast to saturation with drug load increments. Increasing the drug content, mainly leads to an almost proportional increment in the PRO endotherm size leading to a quite linear variation of the PRO enthalpies and a small temperature variation of the PRO endotherm compared with the PRO reference endotherm (Fig. 3 and 4, PRO equation). In the GEL:PIN dispersions, there is a strong interaction between the drug and the carrier even for the higher drug load. With high drug content, the PIN endotherm tends asymptotically to an endothermic curve similar to the GEL:PRO curve. This leads to a more non-linear character of the enthalpy curve in the GEL:PIN systems (Fig. 3 and 4, PIN equation). The drug enthalpies can be described by a quadratic function with a good fit (Fig. 3, Table 1). For PRO, $\Delta H = 0{,}0027.D^2 + 0{,}9974.D - 4{,}6364$ $(R^2=0{,}9991)$ and, for PIN we have the equation $\Delta H=0{,}0109.D^2+1{,}4405.D-17{,}673$ $(R^2=0{,}9983)$, where D and ΔH are the drug load (%) and drug enthalpy (J/g), respectively. Solving these equations for $\Delta H=0$, we get $D_{PRO} \cong 4{,}6\%$, $D_{PIN} \cong 11\%$ which are the limits to measure the drugs enthalpies. Below these values, drugs are likely to be dispersed at a molecular level in the matrix forming a solid solution [4, 5].

Table 1
Data for the enthalpies of the drug and respective endotherms.

Drug (%)	ΔH_{PRO}(J/g)	PRO pk (ºC)	ΔH_{PIN}(J/g)	PIN pk (ºC)
2,5	0	0	0	0
5	1.2	157.1	0	0
10	4.8	157.8	0	0
17	13.6	160.0	10.8	144.7
21,5	16.5	159.9	12.9	148.1
22,5	18.4	159.1	23.3	145.6
25	23.8	159.8	24.9	147.2
35	33.1	159.7	50.5	148.0
50	53.2	160.2	78.5	157.9
60	64.1	160.9	108.7	163.4
100 (1)	122 (2)	163.3	235.9 (2)	168.9

ΔH_{PRO} - Propranolol Enthalpy; ΔH_{PIN} - Pindolol Enthalpy; PRO pk - PRO Endotherm (Peak); PIN pk -PIN Endotherm (Peak)

(1) Reference value for each compound (mean of 3 runs).

(2) ΔH_{max}

Regarding the enthalpies for GEL in both systems, their values are similar for the same drug concentration (Fig. 3 and 4), decreasing on average with drug load, showing that the GEL enthalpy depends more on the drug load than on the type of interaction with the drug or the kind of drug used in this work.

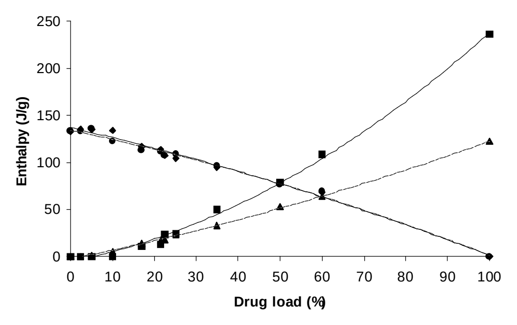

Figure 3 - Enthalpies as a function of drug load.
▲ - ΔH_{PRO} , ■ - ΔH_{PIN} , ● - $\Delta H_{GEL:PRO}$, ◆ - $\Delta H_{GEL:PIN}$

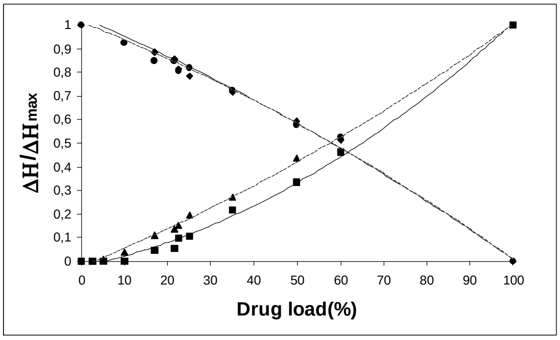

Figure 4 - $\Delta H/\Delta H_{max}$ as a function of drug load.
▲ - ΔH_{PRO} , ■ - ΔH_{PIN} , ● - $\Delta H_{GEL:PRO}$, ◆ - $\Delta H_{GEL:PIN}$

Conclusions

This study has shown that by using calorimetric methods, it is possible to establish relationships between the incorporation of a drug and the matrix, with a better incorporation value of pindolol over propranolol. The results suggest different mechanisms of interaction with the matrix for the two drugs.

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DD-49 CHITOSAN-DNA COMPLEXES FOR GENE DELIVERY

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Introduction

The development of new carrier system for gene therapy represents an enabling technology for treating many genetic disorders. Cationic polymers have the potential for DNA complexation and it is recognised that they may be useful as non-viral vectors for gene delivery. Chitosan is an unacetylated derivative of a natural cationic polysaccharide consisting of repeated glucosamine units. This polymer is biodegradable, biocompatible and low toxic material and able to form polyelectrolyte complexes with DNA. This work carried out studies on the complex formation between plasmid DNA (Ptruff plasmide) and glycol-chitosan and its characterization.

Experimental methods

- **Materials**

Glycol-chitosan (polymerisation degree, min. 400) was purchased from Wako, pure-chemical and DNA (Ptruff plasmide 7Kd) was obtained Dr. Ernesto Marques Júnior (Johns Hopkins University).

- **Preparing chitosan solutions**

Glycol chitosan was dissolved in water (1µg/µL) and sonicated 50W (500W Cole Parmer 501) at different intervals (3, 7, 10 and 15min). These solutions were sterile filtered through a 0.22µm Millipore filter.

- **Preparation Chitosan / Plasmid complexes**

Glycol-chitosan (1µg/µL) was complexated with DNA (Ptruff plasmide) in a phosphate-buffered saline solution of DNA (1µg/µL). The polymer/DNA was 4:1 (w\w). Complexation was visualised in agarose gel (1%, w/v) containing ethidium bromide in Tris-acetate/EDTA (TAE) buffer run at 55 V for 1 hour.

- **Characterization of plasmid / chitosan complexes.**

The size and morphology of plasmid/chitosan complexes were observed using TEM (Transmission Electron Microscopy, JEOL - Japan). A carbon coated 300 mesh copper specimen grid was glow-discharged for 1.5 min. One drop of the complex was deposited on the grid and negatively stained by an aqueous solution of 1% uranyl acetate solution (0.22µm filtered), dried and examined by electron microscope.

Results and discussion

Complex formation between Glycol chitosan and the plasmid was confirmed by electrophoresis of complexes on an agarose gel (figure 1).

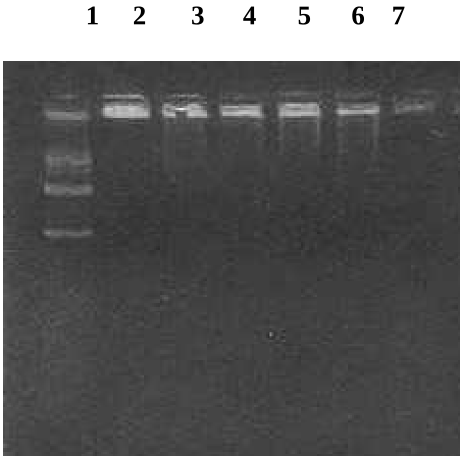

Figure 1. Electrophoresis of Glycol chitosan/plasmid complexes on an agarose gel. DNA was visualized with ethidium bromide. Lane 1, DNA Standard; Lane 2, Glycol chitosan no sonicated + pTruff; Lane 3, Glycol chitosan 3min sonicated + pTruff; Lane 4, Glycol chitosan 7 min sonicated + pTruff; 5 - Glycol chitosan 10min sonicated + pTruff; Lane 6, Glycol chitosan 15 min sonicated + pTruff; Lane 7, Glycol chitosan 7 min sonicated.

A little difference among the samples studied for the complexation process was found. However, there the DNA is practically total self-immobilised to glycol chitosan (line 2), the sonication of the complex release part of the material proportionally to the expose time with different molecular weights.

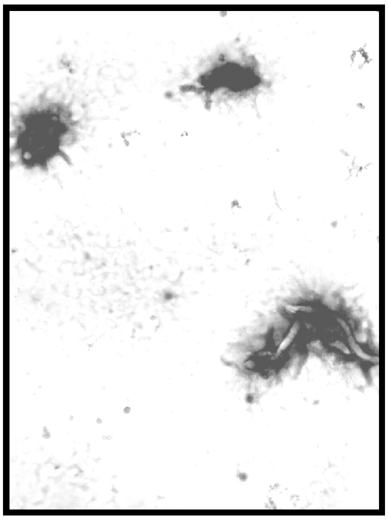

Figure 2 – Transmission electron microscopic TEM observation of Glycol chitosan (1µg/µL) / plasmid (1µg/µL) (4:1) complexes made.

These material can be more suitable for the cell transfection once this behaviour depend on the size and the chemical structure of the polycation complexes. Figure 2 shown the glycol chitosan/plasmid complex in Transmission electron microscopic TEM. The polymer condensed plasmid is shown into donut shape and the size of complexes was ranging from 1000 nm to 2000 nm. The low input of cationic polymer explained the presence of many DNA loops. Furthermore, the electrical potential present in the solution may be suitable to disrupt complexes that were partially condensed. These shapes are similar with those showed by Erbacher, *et al.*, 1998.

Conclusion

This study has shown that glycol chitosan is providing a good polymer to complex plasmid DNA in aqueous media. The self-aggreagtes can form charge complexes when mixed with plasmid DNA. These immobilised systems are considered to be useful for transfer of genes into mammalian cells in vitro and served as a good delivery system composed of biodegradable polymeric materials.

Acknowledgements

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DD-50 A DEVICE FOR ORAL MUCOSAL DRUG DELIVERY

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Introduction

The ecology of the oral cavity depends basically on factors involved in microorganism adherence to surfaces and their nutritional interaction with the host. An alteration in this balance can result in the predominance of pathogenic species, capable of causing disease like dental decay, gingivitis and periodontitis.

The control of the microorganisms causing these diseases has been rendered very difficult due to continuous biofilm formation, a process acting as a permanent source of induction of tooth decay and/or gingival inflammation in the oral cavity [4]. Periodontitis is conventionally treated by scaling and root leveling. However, the formation of niches between dental roots and the capacity of some microorganisms to penetrate connective tissue hindering and sometimes preventing access during the mechanical procedure of scaling, have stimulated the use of drugs for periodontal treatment.

Although good results have been obtained by the use of systemic antibiotic therapy, the bio/mucous adhesive systems used for controlled release of drugs within the periodontal pocket is free from most collateral systemic effects [1]. Therefore, increased use of programmed release systems has become an undisputed reality. Among factors ruling the effectiveness of this type of treatment is the use of an appropriate drug concentration within the periodontal pocket. Generally, the great majority of studies, have demonstrated the efficiency of formulations by examining their microbiological or clinical activity, attested by improvement of gingival health conditions. The aim of this work was the development of a device capable of simulating conditions of the periodontal pocket *in vitro*, reproducing local conditions appropriate to receive a bio-adhesive system for programmed sustained drug release.

Material and Method

A polyethylene jaw, simulating a dental arcade with periodontal pockets, was developed. "In vitro" maintenance of crevicular flow was rendered possible by means of a flexible probe connected to a Luer syringe, filled with artificial saliva, entering the bottom of the dental alveolus. A 0.9µL/min flow speed [3], controlled by a computer system emitting an electric pulse every 60 sec, started an engine inducing rotation of the axis which in turn, propelled the embolus of the syringe, making artificial saliva flow from the bottom of the “periodontal pocket" into the cervical margin of the tooth.

The drug chosen for the study was 15% metronidazole solved in a Pluronic gel vehicle containing 0.2% hydroxypropylmethylcellulose (HPMC) - 400cps [2]. Due to its flowing characteristics at low temperatures, this gel can be easily applied to the inside the periodontal pocket kept at 37ºC. After application, gradual gel formation promotes the final form of the sustained release delivery system, permitting the maintenance of the bioadhesive inside of the niche. Physiological temperature and moisture conditions were reproduced by maintaining the polyethylene jaw model in a thermostatic bath at 37ºC. The artificial saliva flowing from the pocket, was collected at 12 h intervals, and the amount of metronidazole present analyzed by high performance liquid chromatography (HPLC).

Results and Discussion

Confirming the reproducibility of the system, Table I shows that similar patterns regarding volumes collected after 12 h, and concentration of metronidazol per ml were observed in the three identical experimental series performed. This result can also be clearly appreciated in Figure 1.

The data obtained, permit one to conclude that, in the three series tested, approximately the same release profile was observed, figure 1 showing the superposition of the curves during the whole experimental period. In all series, the peak of maximum release of metronidazole occurred during the first 24 hours, then started slowly to decrease, reaching, after 12 days levels still considerably above the minimum inhibitory concentration of metronidazole, of 1.0µg/mL.

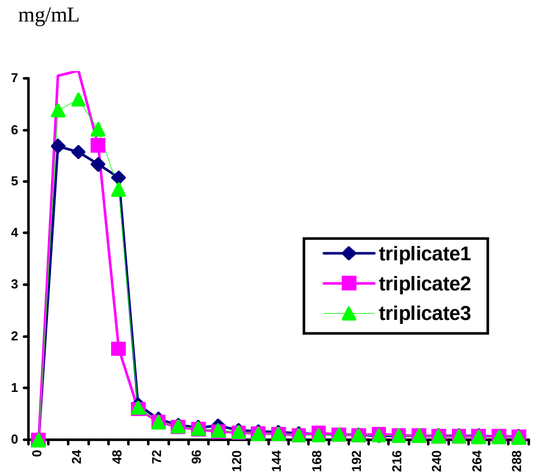

Figure 1 - release profile of triplicates.

Table 1. Volumes collected (ml) and drug concentrations (mg/mL) obtained in a series of three identical experiments

Time	Experiment 1		Experiment 2		Experiment 3	
Hours	mL	mg/mL	mL	Mg/mL	ML	mg/mL
12	0.6	5.690	0.6	7.050	0.6	6.380
24	0.7	5.565	0.8	7.150	0.7	6.585
36	0.7	5.330	0.6	5.692	0.7	6.010
48	0.6	5.072	0.7	1.766	0.7	4.842
60	0.6	0.682	0.7	0.599	0.6	0.621
72	0.8	0.398	0.7	0.351	0.7	0.358
84	0.6	0.292	0.7	0.254	0.6	0.272
96	0.6	0.241	0.8	0.204	0.7	0.223
108	0.6	0.281	0.7	0.161	0.6	0.198
120	0.7	0.182	0.7	0.142	0.7	0.150
132	0.7	0.162	0.7	0.129	0.7	0.120
144	0.7	0.148	0.7	0.120	0.7	0.118
156	0.7	0.127	0.7	0.094	0.7	0.099
168	0.7	0.113	0.7	0.137	0.7	0.095
180	0.6	0.103	0.8	0.101	0.7	0.102
192	0.6	0.101	0.7	0.095	0.6	0.094
204	0.7	0.085	0.6	0.110	0.7	0.095
216	0.7	0.084	0.7	0.087	0.7	0.086
228	0.6	0.078	0.7	0.078	0.7	0.081
240	0.7	0.081	0.7	0.073	0.7	0.079
252	0.6	0.080	0.8	0.067	0.6	0.072
264	0.7	0.077	0.7	0.069	0.8	0.068
276	0.7	0.073	0.7	0.064	0.7	0.062
288	1.0	0.066	0.7	0.062	0.7	0.059

As it is clearly demonstrated in Table 1, the lowest concentration of metronidazole found in the crevicular fluid collected from the “periodontal pocket", was of 59 µg/mL, in the three experiments, and 12 days (288 hours) after application of the gel.

Release of the medication after an attack dose, occurred during the first hours following the insertion of the gel, when it reached a concentration peak in the crevicular fluid, which is considerably above the minimum inhibitory concentration (MIC) of the drug. If we consider that the number of periodontopatogenic microorganisms in an *in vivo* periodontal pocket is very high, this fact is probably beneficial for antibiotic therapy.

Conclusion

The equipment developed, revealed itself appropriate for the *in vitro* study of controlled drug delivery in periodontal pockets, simulating conditions of constant temperature and crevicular fluid flow and within an expected pattern. The device should be optimized according to the number of niches. Concerning the Pluronic gel, we consider that it propitiated the release of therapeutic levels of metronidazole which were maintained during the twelve days of the experiment.

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DD-51 EFFECT OF THE VEHICLE COMPOSITION ON THE RELEASE OF KETOPROFEN

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Introduction

The effective absorption of drugs through the skin has encouraged the development of transdermal delivery systems with the benefits of decreasing the administration frequency and the risk of side effects commonly found with other routes of administrations. For a drug elicit a systemic action by application on the skin surface, must first diffuse out of the vehicle onto the skin surface and then it must permeate this natural barrier to reach the site of action. These processes are dependent upon the physical properties of the drug, the vehicle and the barrier [1]. The selections of the appropriated vehicle are very important to increase the efficacy of transdermal products. A topical vehicle can affect the percutaneous penetration of a drug by modifications of the permeability of the skin barrier and by releasing the active principle in significant amount and adequate rate. Ketoprofen is a non-steroidal anti-inflammatory drug that is used in the treatment of rheumatoid arthritis. This drug can be used as a suitable model to develop transdermal formulations because of its short half-life of about 2 hours and gastrointestinal side effects [2-4].

Objective

The aim of this work was to evaluate the influence of the vehicle composition on the release kinetics of ketoprofen. For this purpose, two gels of hydrophilic matrices (methylcellulose and carbomer) and two oil-in-water emulsions of different HLB values were formulated.

Methodology

Gels of methylcellulose (MC) and carbomer (CAR) were formulated with an approximate viscosity of 25000 mPas and pH 5.5. In these hydrophilic matrices, the thermodynamic activity of ketoprofen was maintained constant by using a saturated system in the presence of excess solid drug. Two oil-in-water emulsions of different HLB values (12 and 4.6) were prepared using Tween 60 and Span 80, as emulsifying agent, in appropriate proportions. Mineral oil, cetyl alcohol and stearic acid were the oil phase. The water phase was containing conservant, water and drugs (1%).

The drug release experiments were performed in Franz diffusion cells with a semi-permeable membrane of cellulose acetate. The receptor fluid, distilled deareated water, was maintained at 37ºC, and stirred with a magnetic stirrer. From the receptor compartment, aliquots were withdrawn periodically for 8 h, and replaced with an equal volume of receptor phase. The ketoprofen content was analyzed spectrophotometrically at 260 nm.

Results are presented as mean ± SD (n=6). Statistical analysis was performed by ANOVA and Duncan tests; $p<0.05$ was considered significantive [5].

Results

Tables 1 and 2 show the composition of the emulsions and physicochemical parameters of the formulated vehicles, respectively.

Table 1
Composition of formulated emulsions.

COMPOSITION (g/100 g)	**HLB 4.6**	**HLB 12**
Ketoprofen	1	1
Mineral oil	15	15
Cetyl alcohol	3	3
Stearic acid	4	4
Span® 80	9.7	2.7
Tween® 60	0.3	7.3
Water c.s.p.	100	100

Table 2
Physicochemical parameters of formulated vehicles.

VEHICLE	pH	VISCOSITY (mPas)
MC	5.52	24500
CAR	5.55	25250
HLB 4.6	5.56	21000
HLB 12	5.69	10000

The drug/polymer ratio of methylcellulose and carbomer gels was 0.26 and 1.44, respectively.

The release of ketoprofen from both gels fixed to the model of the square root of time. The release kinetics of ketoprofen from the emulsions was fitted to a zero order model.

Table 3
Amount of ketoprofen released at 8 hours and release rate from gels ($mg/cm^2\ h^{½}$) and emulsions ($mg/\ cm^2\ h$) of different HLB value.

VEHICLE	RELEASED % at 8 h	RELEASE RATE
MC	65.6 ± 0.8*	0.51 ± 0.01 *
CAR	92.0 ± 2.2*	0.68 ± 0.06 *
HLB 4.6	39.8 ± 0.7*	0.12 ± 0.01 *
HLB 12	22.4 ± 0.4*	0.07 ± 0.01 *

* significative difference between vehicles ($p<0.05$).

Table 3 shows the apparent release rate constant of ketoprofen and the % released at 8 hours from the formulated vehicles. The release profiles of ketoprofen from the formulated vehicles are shown in figure 1.

The drug release rate from the gels was higher from carbomer ($p<0.05$). The polymer charge and polarity modify the release rate of ionic drugs [6]. From the emulsions, the ketoprofen release rate was higher with 4.6 HLB value ($p<0.05$).

Emulsion with the lowest HLB value contains less proportion of hydrophilic emulgent. Decrease of Tween 60 concentration causes no micellar solubilization of ketoprofen, increasing the thermodynamic activity of the drugs in the aqueous phase.

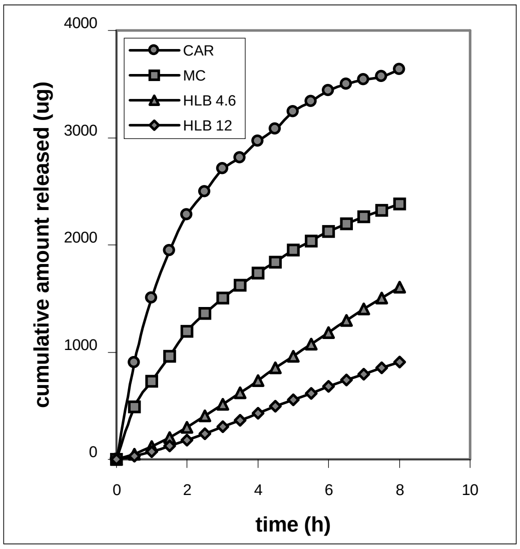

Figure 1. Release profiles of ketoprofen from gels and emulsions.

Conclusion

The results show that the release of ketoprofen from the vehicles evaluated is produced to a constant and controlled release rate. Both, gels and emulsions may be useful as vehicles for the transdermal administration of ketoprofen and other drugs with optimal lipid/water partition coefficient.

Acknowledgements

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BI-01 EXTRA/INTRA-VASCULAR SUBSTANCE CONCENTRATION RATIO TO MEASURE THE CARDIAC OUTPUT DISTRIBUTION AS AN EXPERIMENTAL APPROACH:PRELIMINARY RESULTS

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Introduction

Many are the functions of the human body circadianally affected, that alter the fate of substances in the organism. The cardiovascular system as means of rapid connection among organs rules the stages of liberation, absorption and disposition, and it might be the cause of alteration of intra- and extra-vascular drug levels [1]. In consequence, a cardiac output redistribution would generate changes in drug concentration in the action sites, mostly located in the extravascular zone [2]. The input rate of a substance from systemic circulation to biophase depends on the fraction of blood flow that is destined to it, originating a drug concentration relationship between both spaces whose value is a function of hemodynamic changes.

It is of interest to investigate the cyclic variations of cardiac output distribution through a procedure of simple execution. It would seem suitable the use of a substance of particular features for the determination of the relationship tissue/plasma drug concentration ratio. In this research the extravascular fluid for sample monitoring is saliva. The diffusible solute concentration in saliva from capillary plasma is the result of a continuous exchange in the glandular conducts that is altered by an increase in secretion rate [3]. The drug tissue level, as a result of the established equilibrium, can be quantified in a first saliva fraction, while through induced and continuous salivation, fractions whose composition is similar to free plasma concentration are progressively obtained [4]. The collection of saliva samples in two consecutive fractions, S1 and S2, that would reflect the tissue and capillary composition respectively, would constitute a simple procedure.

The saliva has the advantage of being a practical and non-invasive sampling fluid. The characteristics of the tracer substance employed, paracetamol, of optimum quantification in this fluid, broad therapeutic range and of which no cardiovascular action has been described yet, suggests it would be the right one for performing this procedure.

This research intends to study how sensitive the tissue/plasma paracetamol concentration ratio or S1/S2 is to variations of the cardiac output distribution.

Experimental method

In a first experience, a healthy volunteer is subjected to two physiological situations at different hours of the day: rest (30 minutes sitting) and exercise (15 minutes walking with a steady intensity following the same route). Two 500 mg paracetamol tablets were taken, the first one at 24:00 prior the day of the experience and the second one at 12:00 of the day of the experience. The chosen hours for sampling in rest were: 08:00, 12:00, 16:00, 20:00 and 24:00, and in exercise: 08:15, 12:15, 16:15, 20:15 and 24:15. At each hour, the stimulated saliva with citric acid over the tongue, was taken in two fractions S1 and S2, both in an amount of 1 ml, taking care saliva total emission time did not exceed one minute. The fractions were neutralized with an equivalent amount of sodium bicarbonate, previously weighed in a collector tube.

In a second experience the same volunteer was subjected to 10 minutes of controlled exercise on a fixed bicycle at a medium intensity strength and at a speed of 28 km/h. Both S1 and S2 samples were obtained five and ten minutes after the exercise started at the following hours: 04:00, 10:00, 16:00 and 22:00 (all of them 4 hours post 500 mg dose).

The salivary quantification of paracetamol was performed by an HPLC method using a reversed phase column (C18), 3-acetomidophenol as the internal standard and following an analytical protocol previously described [5], with minor validated modifications. To 0.5 ml of saliva, 50 µl of internal standard (52.5 mg/L) and 2 ml of ethylacetate were added, subsequently shaken with a vortex for 1 minute and centrifuged for 5 minutes at a speed rotation of 3000 rpm. The organic phase was evaporated to dryness under flowing air at 37°C. The residue was reconstituted with 100 µl of methanol and 20 µl were injected onto the column. The HPLC chromatographic system was: mobile phase $H_2O:CH_3CN$ (92:8), 2ml/min flow rate, 242 nm wavelength and room temperature.

Results and discussion

The results of the first experience showed the following S1/S2 ratios: at rest 1.12 (08:00), 0.96 (12:00), 1.13 (16:00), 1.13 (24:00); at exercise 1.37 (8:15), 1.28 (12:15), 1.20 (16:15), 0.98 (20:15), 1.05 (24:15). A regression analysis S1/S2 vs. time showed no linear correlation during rest, obtaining ratio values almost constant. In contrast after 15-minute exercise a significant linear correlation ($p<0.025$), between S1/S2 vs. time, was obtained. The morning exercise S1/S2 ratios were higher to those obtained in the afternoon and at night.

It is widely known that sympathetic activity is dependent on the time of the day, being higher in the morning than in later hours [6]. This could be reflected in the variation of the S1/S2 ratio shown in Figure 1 after exercise was performed according to the second experience, but not after the rest period. It seems logical to think that the impact of some action over a system could be better observed when the system is operating at its maximum capacity: the highest performance in the morning hours and the lowest performance in the evening hours. An adrenergical stimulus, increased in exercise, originates a forced cardiac output redistribution to the extra-splanchnic region (ESR) (where muscle and saliva glands are included) at expense of a splanchnic-renal (ER) loss of blood according to a smaller diameter of the vessels there located.

Likewise, for 5-minute exercise, higher S1/S2 ratios were found at 04:00 and 10:00 than 16:00 and 22:00, unlike the 10-minute exercise where S1/S2 ratios were closer to rest values. Literature [7] contributes with interesting data about the regulation of muscle blood flow and cardiac output. At the beginning of muscle activity, there is an immediate, massive sympathetic discharge producing fast circulatory readjustments of which one of the most important is a strong ER vasoconstriction that favors redistribution of blood flow to the ESR. In another stage, because an increase of physical activity throughout time, a higher local metabolic demand produces a larger caliber of muscle vessels. This blood flow fraction increase in the muscles seems to be the cause for a lower S1/S2 ratio, even to lesser extent than that of rest.

Conclusion

It could be stated that during exercise there is a reorganization of the cardiac output fraction which could affect the S1/S2 ratios. Even though these results were taken from a single subject they are in accordance with well-known events of human physiology. This suggests a promising future for this procedure. In further studies, research will be done over a large number of subjects undergoing the same exercise and in other physiopathologic situations.

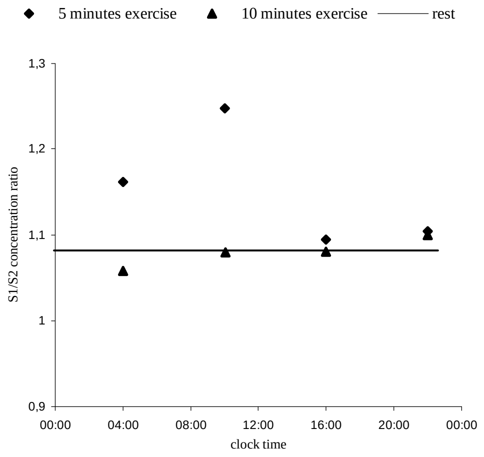

Figure 1. Paracetamol S1/S2 concentration ratio as a function of time. Data from first (rest shown as an average) and second experience (5 and 10 minutes exercise) are shown together.

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BI-02 PLASMA DRUG MONITORING IN BIOEQUIVALENCE ASSESSMENT: HOW ACCURATE IS IT FOR VARIABLE DRUG-INDUCED PHARMACOKINETIC SYSTEMS?

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Introduction

Caffeine is a widely used drug not for medical purposes but included in current beverages and food. Despite its limited use in therapy, mainly associated with analgesic drugs given orally or in newborn apnea, its pharmacokinetics is well known. For this reason, caffeine is a good example for the purpose highlighted in the objective.

A linear decrease of the apparent first-order elimination rate constant with dose (50 to 750 mg) was reported [1], and there was a trend towards increasing apparent volume of distribution with increasing dose. However, the bioavailability and the total body clearance were unaffected by dose. Besides, the saliva/plasma (S/P) caffeine concentration ratio increased with dose. Our research group not only agrees with these observations but also adds some more details: linear system in plasma and non-linear system in saliva drug monitoring were observed when caffeine dose was increased from 100 to 400 mg (confidential and non-published data). Otherwise, the S/P ratio was affected by dose regarding samples that belong to a period of time comprising between 4 and 12 hours post-dose. The 24-hour-cumulative urine volumes decreased with increasing dose.

Other investigators [2] observed a significant positive correlation between thermogenic response and caffeine plasma concentration (dose) due to an increase of vascular smooth muscle tone. Two different reports [3, 4] showed a S/P ratio 30% higher than the caffeine plasma free fraction, with dose ranged from 200-350 mg.

All these experimental results enable us to postulate the following scenario for caffeine disposition. At lower concentrations in the organism (up to 100 mg of dose) the drug acts as vasodilator [5]. Conforming the dose increases the drug level does too. Gradually, its concentration at the central nervous system (CNS: vasomotor center) starts to activate the sympathetic tone. So, renal perfusion decreases and the blood is carried to other regions of the body without affecting liver blood flow in order to maintain a constant clearance of the drug (hepatic pathway as the main route of elimination). This process could be of a positive feedback way involving dose near 200 mg.

The objective of this study is to evaluate whether the bioavailability parameter estimated from different body fluids could affect the conclusions on bioequivalence, for a drug which is able to modify its disposition.

Method

Taking into account our hypothesis we have carried out a simulated bioequivalence trial with a dose of 200 mg, using a hepta-compartment model described in another paper at this same Congress. We have considered only two pharmacokinetic systems; one of them was operative at lower concentrations and the other one at concentrations higher than 2.5 mg/L in the biophase that included the CNS. This level was set arbitrarily but it was suspected to be present for that dose, according to our experimental results and to other data previously published [1]. The model is shown in Figure 1, which displays different zones of the body. Pharmacokinetics parameters obtained experimentally with dose of 100 mg according to a classical two-compartment model were used to feed the simulation program. The change of system assumed a decrease in the cardiac output fraction served to the renal region of 45% and an increase of 30% for blood flow fraction served to the extra-splanchnic-renal regions, when the drug concentrations exceed 2.5 mg/L.

The bioequivalence trial was performed with 12 subjects in a two- treatment (brands A and B) and a two-period crossover design. Brands were of 200 mg as nominal dose, having form A and form B a bioavailability fraction of 0.95 ± 15 % and 0.85 ± 15 % respectively. The absorption rate constant was kept as 2.55 h^{-1} , and neither the period nor the interindividual variability for caffeine dispositions were considered in the simulations. Area under the concentration-time curve (AUC) and maximum concentration (Cmax) for compartments 1 (plasma), 1b, 1c and 2c were estimated according to the trapezoidal rule and the “ experimental “ value issued by the program, respectively. These bioavailability parameters were processed by ANOVA test followed by the two one-side test of the ratio of means (bioequivalence range of 0.8-1.25 was set). Mean values for the parameters and the intraindividual coefficient of variation (CV) were computed.

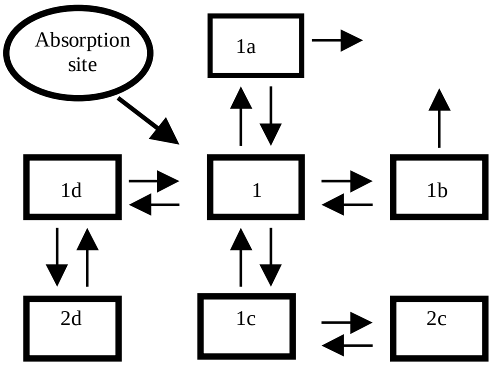

Figure 1:
1: Intravascular compartment (plasma),
1a: Splanchnic zone, 1b: Renal zone, 1c and 1d: Extra-splanchnic-renal regions, 2c: Biophase in the peripheral compartment, 2d: Other peripheral compartment.

Results

For compartments 1, 1b, 1c and 2c the form B / form A ratio of AUC means ± CV were : 0.88 ± 8.6%, 0.95 ± 7.7%, 0.84 ± 11.4% and 0.82 ± 9.3% respectively. Cmax mean ratios were practically the same for compartments 1, 1b and 1c (0.89), being 0.83 for compartments 2c. If the conclusion of bioequivalence were taken from plasma results (compartment 1), forms A and B would be assessed as bioequivalent. The same would be concluded from compartment 1b. But, on the contrary, forms A and B would be bioinequivalent if compartments 1c (because of AUC) and 2c (because of AUC and Cmax) were considered in the statistical analysis.

Discussion and Conclusion

The most important fact was the bias obtained for the ratio of means. The actual difference between brands (10 %) was respected in plasma but biased in the renal zone (1b, diminished) and in the extra-splanchnic-renal regions (1c, 2c, increased). In the experiment, the ratio of means dropped within the bioequivalence range but the forms were bioinequivalent in compartments 1c and 2c, due to statistical reasons. If brands A and B had a more bioavailable dose difference (but below 20%), also the AUC mean ratios would be outside the bioequivalence range. In fact, this is not only a quantitative but also a qualitative problem. Two drug products are bioequivalent if they have similar drug effect and so, similar drug concentration at the action sites. The question is: where are the action sites? – Certainly they are not in plasma for most drugs.

In conclusion, for some cases the bioequivalence trials using plasma as biologic fluid would miss the actual drug bioavalability as it is conceived therapeutically. Drugs acting over the cardiovascular system, directly or indirectly, could present this duality when concluding bioequivalence.

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BI-03 COMPARTMENTAL MODEL TAKING INTO ACCOUNT INTRAVASCULAR AND EXTRAVASCULAR SITES

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Introduction

Classical pharmacokinetic models divide the body in several compartments in which the drug concentration homogenizes instantaneously. After drug administration, the substance enters the central compartment, that includes blood and some parts of extravascular spaces where the drug concentrations are quickly equilibrated with the plasma ones. From this first compartment the substance diffuses towards other compartments called "peripherals". Frequently the exit of a drug is supposed to be held from the central compartment.

But, we could consider another possibility than a simple two-compartment classical model. Instead of only one central compartment, an intravascular compartment (IVC) and several extravascular compartments (EVCs) independently connected with the former would get the equilibrium in a very short time, if there was not any mass exchange except between them. From the IVC the drug is distributed to the EVCs according to the cardiac output rate, but the mass exchange in the direction IVC ► EVC takes into account the cardiac output fraction served to each zone of the body. The input of drug takes place from the absorption site into the blood (IVC). The classical peripheral compartment belongs to the body but not necessarily located in a specific zone. For that reason, all or some EVCs of the new model are connected with parts of the peripheral compartment (PC). The exit of drug when this was classically supposed to be held from the central compartment, now takes place from one or more EVCs. To sum up, the substance transfer to the exterior and/or deeper compartments (peripheral) is made from IVC through EVCs. In this context the mass exit from IVC (i.e. plasma) is governed by the cardiac output fraction served to each region according to the diameter of vessels of the circulatory system.

Methodology: An Example With Paracetamol

With this idea we have designed ahepta-compartment model with 1 IVC, 4 EVCs and 2 PCs. All kinetic processes were of first-order. The exit of drug from the body took place from 2 EVCs located in the splanchnic-renal (SR) region . Even though the PCs could be placed anywhere, we have linked them to each remaining EVCs located in the extra-splanchnic-renal (ESR) region. The fourth-order Runge-Kutta method was used to calculate the amount of drug in each compartment throughout time. Pharmacokinetic parameters of Paracetamol according to a two-compartment classical model published previously (1) were used to feed the program, which was written on the Mathcad Plus 8.0 software for Windows.

Those classical parameters: K_{12} = 0.273 h^{-1}, K_{21} = 0.496 h^{-1}, K_{10}renal = 0.011 h^{-1} , K_{10}nonrenal = 0.352 h^{-1}, V_1= 44.0 L, V_2= 24.2 L were transformed into the new ones taking into account the following assumptions:

1. The same renal, non-renal, central to peripheral and peripheral to central clearances shown in the classical model were kept.
2. The cardiac output distribution towards the SR and ESR regions were 40% (22% splanchnic plus 18% renal) and 60% (18% plus 42%) respectively.
3. At the steady-state all the compartments had identical drug concentrations.
4. All transference processes returning drug to the IVC had the same rate constant. Similarly, all rate constants involved with mass transfers from EVCs to PCs had the same value. The rate constants for the direction PCs ► EVCs were identical to K_{21}.
5. The IVC volume was set as 6 L.
6. The whole exit of drug from the IVC had a rate constant of 60 h^{-1}.
7. The volumes of each EVCs were proportionally related to the cardiac output distribution served to each zone, considering a total volume of (44 - 6)L .

Classical model and new model simulations were performed with a dose of 1000 mg administered at:

08:00, bioavailability fraction of 0.88, and absorption rate constant of 2.5 h^{-1} (constant system). A simulation was also made with the new model changing the ESR/SR cardiac output distributions in a 24-hour-cycle way: 66/34 (08:00-14:00), 60/40 (14:00-20:00), 54/46 (20:00-02:00) and 60/40 (02:00-08:00) (variable system).

Results

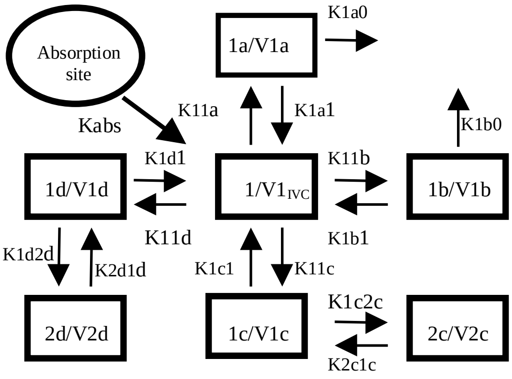

Figure 1: $V1_{IVC}$ =6.0 L, V1a =8.4 L,V1b =6.8 L, V1c =6.8L,V1d =16.0 L,V2c=7.3 L,V2d =17.0 L, K1a1=K1b1=K1c1=K1d1=9.5 h^{-1},K11a =13.2 h^{-1}, K11b =10.8 h^{-1}, K11c =10.8 h^{-1}, K11d =25.2 h^{-1}, K1c2c =K1d2d =0.5 h^{-1}, K2c1c =K2d1d =0.5 h^{-1}, K1a0 =2.6 h^{-1},K2b0= 0.1 h^{-1},Kabs= 2.5 h^{-1}.

The model with compartment volumes and rate constants are shown in Figure 1. Absorption site, IVC, EVCs and PCs are visualized as a circle and squares numbered with 1, 1a, 1b, 1c, 2c and 2d respectively. Figure 2 shows simulated Paracetamol concentrations in the IVC (plasma) and the 1c/1 drug concentration ratio throughout 24h post-dose (variable system).

Discussion and conclusions

Predicted plasma concentrations from the classical two-compartment model and from the new hepta-compartment model were superimposed, when the system was constant , making the new approach consistent. This was reached by iterative assignment of IVC volume (for this case the volume was 6 L). Changes of the cardiac output distribution yielded some pharmacokinetic feature, which were different from that obtained with the 60/40 ESR/ER blood flow ratio (constant system). For instance, non-equal drug concentration among "central" compartments (IVC and EVCs) were observed, a higher half-life when blood flow decreased to the elimination zones, etc..

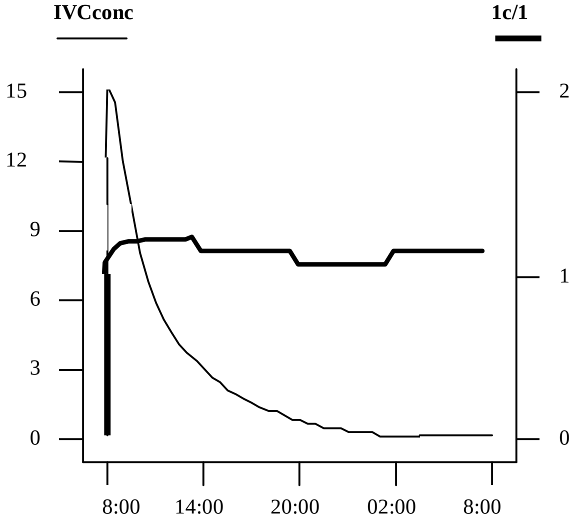

Figure 2: ——— Simulated Paracetamol concentrations in plasma (mg/L): **IVCconc.**; ▬▬ 1c/1 drug concentration ratio:**1c/1**

As a conclusion, the cardiac output distribution would have serious impact on drug concentrations in the body, not always well predicted from the plasma drug concentrations.

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BI-04 STUDY OF CONVULSION MODEL WITH PILOCARPINE IN YOUNG AND ADULT RATS: BEHAVIORAL AND NEUROCHEMICAL ALTERATIONS

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Introduction

Several researches have seen done using high doses of pilocarpine (400 mg/Kg) in order to characterize this model of convulsion, investigate pathophysiology and identify other therapeutics agents to epilepsy treatment, some drugs as melatonin, glutamate, apomorphine well as plants, have been studied using this model.

In fact, high doses of this muscarinic cholinergic agoinst, administered systemically result in behavioural changes, seizures and widespread brain damage in adult rats. The treatment results in epileptics states and death in younger animals. However, in the latter group, brain tissue examination demonstrates less intense cerebral damage suggesting that histopathological changes are an age related phenomenon.

It is well demonstrated that convulsions induced by pilocarpine can be blocked by prior use of atropine, consistently with the involvement of the cholinergic system. It is not established whether other neurotransmitters play a role in maintenance of convulsions and development of cerebral changes. Little is known about changes in neurotransmitter concentrations during the pilocarpine seizures induced.

The goals of the present work were to study, in striatum of young and adult rats, the behavioural and neurochemical alterations induced by high doses of pilocarpine in order to further clarify this model to investigate new drugs in epilepsy.

Experimental methods

- **Treatment of animals**

Wistar rats (250-280g, 2 months old) were injected subcutaneously (sc) with saline (controls) or single dose of pilocarpine cloridrate (400 mg/Kg, P400). Younger animals (30-35g, 21 days old) were submitted to the same treatment. Animals were closely observed for behavioral changes immediately after the pilocarpine injection during 24 h and after they were sacrificed and their brains were removed for neurochemical study.

- **Determination of Neurotransmitters Levels**

For determination of monoamine levels, 10 % striatum homogenates were sonicated in 0.1M $HClO_4$, for 30 seconds and centrifuged at 4° C for 20 minutes at 26.000g. A 20µL sample of the supernatant was then injected in the high-performance liquid chromatograph (HPLC) column. The mobile phase was 0.163M citric acid, pH 3.0 containing 0.02 mM EDTA, with 0.69 mM sodium octanesulfonic acid (SOS), as ion pairing reagent, 4% v/v acetonitrile and 1.7% v/v tetrahyrofuran. Concentrations of dopamine (DA), serotonina (5-HT), as well as their metabolites (3,4 dihydroxyphenileacetic acid – DOPAC, homovalinic acid – HVA, 5-hidroxyindoleacetic acid – 5-HIAA) in rat striatum submitted to a single dose of pilocarpine (400 mg/Kg, sc) were electrochemically detected using amperometric detector (Shimadzu, Japan), by oxidation on a glassy carbon electrode at 0.85 v realive to the Ag-AgCl reference electrode. The results were expressed as ng/g of tissue.

- **Statistical analysis**

For statistical analyses, ANOVA was used followed by Student's t-test. The difference with $p<0.05$ was considered statistically significant.

Results and discussion

Peripheral cholinergic reactions, such as miosis, piloerection, chromodacriorrhea, diarrhea, and stereotyped movements (continuous sniffing and paw licking) were observed in all animals at both ages, after P400 treatment. Convulsions prossing to epileptics status developed in 75% and 81% of young and adult animals, respectively, which also presented a high percentage of death. In younger group, the number of convulsion was little tan in adult animals (table1).

In our work a significant decrease in DA and 5-HIAA levels was observed in striatum from rats submitted to pilocarpine-induced convulsions when compared to controls in 21-day-old rats. A tendency for decrease in 5-HT was seen too (table2). In 2-month-old rats, a concentration of DA, 5-HT and HVA was significantly decreased, as compared to control group (table3).

Pilocarpine administration has been used as an animal model for temporal lobe epilepsy since it produces several morphological and synaptic features in common with human convulsions. Smolders *et al.* em 1997, observed that during the subsequent pilocarpine-induced limbic convulsions extracellular glutamate, GABA and DA levels in hippocampus were increased, suggesting neuronal vesicular release, however more studies including others brain areas should be done to identify the importance of monoamines in the epileptic phenomenon. Ontogenic studies show an age-dependent difference in response of animals treated with pilocarpine. It might be connected to selective maturation of neural systems essential to epileptogenesis and brain damage.

Table 1: Behavioral Alterations observed in younger animals of 21 days and adults 2 months old treatment with pilocarpine.

Behavioral Alterations*	***P400* *21 days-old***	***P400* *2 month-old***
Peripheral cholinergic signs**	100	100
Tremors	100	50
Stereotyped Movements***	100	100
Convulsion	100	75
Status Epilepiticus	81	75
Death	56	58
No. Animals	16	12

Rats Wistar (250-280g, 2 months old) and (30-35g, 21 days old) were treated with a single dose of pilocarpine (400 mg/Kg, sc.). The animals were submitted to a 24-h observation.

* percentage of animals presenting behavioral alterations.

Peripheral cholinergic signs**: miosis, piloerection, chromodacriorrhea, diarrhea, and stereotyped movements (continuous sniffing and paw licking).

Table 2: Alterations of the levels of monoamines and metabolites striatum of young rats of 21 days treatment with pilocarpine.

MONOAMINES	***Control* *21 days***	***P400* *21days***
DA	3088±210.6 (6)	1614±143.1(6)*
DOPAC	849±34.2 (7)	814±151.7 (6)
5-HT	778±186.1 (6)	360±46.5 (6)
5-HIAA	1160±218.7 (6)	455±39.0 (7)*
HVA	578±82.6 (8)	689±75.4 (4)

Data are reported as (means ± SEM) for the number of animals shown in parentheses. For statistical analyses, ANOVA, and the Student’s t-test were used.

* $p<0.05$ compared to control.

Table 3: Alterations of the levels of monoamines and metabolites striatum of rats adults 2 months old treatment with pilocarpine.

MONOAMINES	***CONTROL* *2 months***	***P400* *2 months***
DA	3268±251.0 (19)	1717±60.5 (5)*
DOPAC	2373±179.8 (25)	1826±268.9 (5)
5-HT	463±24.2 (15)	325±63.3 (6)*
5-HIAA	588±40.1 (15)	492±87.2 (5)
HVA	1047±58.6 (21)	760±127.7 (5)*

Data are reported as means ± SEM for the number of animals shown in parentheses. For statistical analyses, ANOVA, and the Student t-test were used.

* $p<0.05$ compared to control.

Conclusion

The susceptibility to pilocarpine-induced seizures in rats increases with age.

Our work showed that DA levels decrease in both ages, suggesting a role for this catecholamine in convulsive process. 5-HT appears to be involved too.

The knowledge about neurotransmitters that have a participation in convulsions is important to establish strategies to investigate new drugs in epilepsy.

Acknowledgements

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BI-05 EVIDENCE FOR SIMILAR NUMBER OF TITYUSTOXIN BINDING SITES IN DIFFERENT ORGANS OF ADULT AND WEANLING RATS

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Introduction

The signs and symptoms of scorpion envenomation have been accepted to be due to properties of neurotoxins that bind to voltage dependent sodium channels in excitable membranes [1]. Children present higher susceptibility to the scorpion venom than adult [2,3,6]. The higher sensitivity of children could be due to the concentration of the venom in the circulating blood because the lower body weight or changes in the number and distribution of binding sites during acute phase of organism development.

Objective

The objective of this work was to show evidence that the number binding sites does not vary in adults and weanling rats, using the toxin (TsTX) from *Tityus serrulatus* venom, labeled with ^{99m}Tc.

Methodology

Adult (150-160 days) and weanling (21-22 days) Holtzman rats weighing respectively 353 ± 33 g and 38 ± 6 g were used. The tityustoxin (TsTX) was obtained according to [5] and labeled with ^{99m}Tc following the procedure of PAUWELS *et al.* [4]. Subcutaneous via was used to inject the toxin at the dose of 6 μg/animal. Animal groups were sacrificed after anesthesia with i.p. injection of urethane (140 mg/100 g body weight), at 5, 60 minutes and 3, 6, 12, and 24 hours and the following organs were excised: brain, heart, thyroid, lungs, spleen, liver, kidneys. The organs were washed, dried in filter paper and weighed. The radioactivity was measured and expressed as a % of the dose/ organ, considering 100% the radioactivity found in the exact amount injected. Statistical analysis was done by t-test ($p<0.05$).

Results and Discussion

The results indicate that the uptake per organ for similar amount of toxin, injected s.c. in adult and weanling rats, differs in most the studied time, being higher in the younger rats.

Table 1

Distribution of ^{99m}Tc-TsTX (%dose / organ) in adult rats (AR) and weanling rats (WR). Data are reported as the mean ± SE (n = 5-8)

		Time (minutes)					
Organ		**5**	**60**	**180**	**360**	**720**	**1440**
Brain	AR	0.001 ± 0.0003	0.004 ± 0.0006	0.008 ± 0.0007	0.001 ± 0.0004	0.001 ± 0.0004	0.00 ± 0.00
	WR	0.01 ± 0.002*	0.03 ± 0.004*	0.02 ± 0.002*	0.03 ± 0.003*	0.01 ± 0.001*	0.02 ± 0.008*
Heart	AR	0.01 ± 0.002	0.05 ± 0.007	0.07 ± 0.003	0.02 ± 0.001	0.01 ± 0.001	0.005 ± 0.003
	WR	0.05 ± 0.006*	0.07 ± 0.009*	0.05 ± 0.009	0.05 ± 0.004*	0.02 ± 0.001*	0.01 ± 0.004*
Thyroid	AR	0.002 ± 0.0005	0.02 ± 0.003	0.03 ± 0.004	0.008 ± 0.0005	0.003 ± 0.0007	0.002 ± 0.0007
	WR	0.007 ± 0.0008*	0.03 ± 0.003*	0.02 ± 0.001*	0.02 ± 0.001*	0.009 ± 0.0006*	0.005 ± 0.004*
Lung	AR	0.03 ± 0.007	0.13 ± 0.01	0.30 ± 0.04	0.09 ± 0.01	0.06 ± 0.007	0.06 ± 0.03
	WR	0.16 ± 0.05*	0.35 ± 0.04*	0.19 ± 0.03*	0.22 ± 0.01*	0.07 ± 0.004	0.08 ± 0.006*
Spleen	AR	0.003 ± 0.0006	0.03 ± 0.006	0.08 ± 0.01	0.05 ± 0.003	0.02 ± 0.003	0.03 ± 0.006
	WR	0.04 ± 0.007*	0.06 ± 0.005*	0.06 ± 0.02*	0.08 ± 0.003	0.04 ± 0.002*	0.04 ± 0.007*
Liver	AR	0.14 ± 0.03	0.65 ± 0.06	1.85 ± 0.15	0.82 ± 0.05	0.91 ± 0.06	0.53 ± 0.06
	WR	0.43 ± 0.08*	1.30 ± 0.07*	2.98 ± 0.89	2.11 ± 0.16*	0.76 ± 0.04*	0.69 ± 0.05*
Kidney	AR	0.09 ± 0.01	6.90 ± 0.92	32.35 ± 3.65	38.37 ± 2.50	11.31 ± 0.37	25.52 ± 1.60
	WR	0.45 ± 0.04*	7.96 ± 0.22	18.62 ± 0.91*	41.61 ± 1.02	17.00 ± 0.68*	19.19 ± 0.52

*($p<0.05$)

The kidney was the organ that showed higher uptake for both ages, with a maximum at 360 minutes. It was observed that the maximum content for the lungs, heart, spleen, thyroid, and brain were reached quicker in the young rats (60 minutes vs. 180 minutes). However, the maximum contents per organ did not differ statistically between adult and weanling rats, except for brain where the values were 3.8 greater for young animals (Table 2).

Similar maximum content in these organs indicates that the number of binding sites is the same in both ages studied. Considering the organ weight in both ages, one can suggest that the relative concentration of binding sites is higher in young rats.

Table 2

Maximum uptake (C_{max}) of ^{99m}Tc-TsTX (% dose / organ) in adult and weanling rats. Results expressed as mean ± SE (n = 5-8).

	Adult rats		**Weanling rats**		**test t**
Organ	**C_{max}**	**$T_{max(min)}$**	**C_{max}**	**$T_{max(min)}$**	**p<0.05**
Brain	0.008 ± 0.0007	180	0.03 ± 0.004	60	*
Heart	0.07 ± 0.003	180	0.07 ± 0.009	60	ns
Thyroid	0.03 ± 0.004	180	0.03 ± 0.003	60	ns
Lung	0.30 ± 0.04	180	0.35 ± 0.04	60	ns
Spleen	0.08 ± 0.01	180	0.08 ± 0.003	360	ns
Liver	1.85 ± 0.15	180	2.89 ± 0.89	180	ns
Kidney	38.17 ± 2.50	360	41.61 ± 1.02	360	ns

*(p<0.05); ns: not significant

Conclusion

The formation of toxin binding site precedes the full growth of the organs (except for brain). The evidence is in the fact that there is no difference in maximum content of ^{99m}Tc-TsTX in adults and weanling rats in all studied organs, suggesting that the number of total binding sites does not differ with the studied ages. In brain, however, the higher uptake in young rats indicates a possible passage of the toxin through blood brain barrier what does not seem to happen in adult rats. If we consider the fact that the organ size is different in both groups, it is reasonable to suppose higher concentration of binding sites in young animals, which might explain the seriousness of scorpion accidents in children.

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BI-06 THE LIPID-LOWERING EFFECT OF *TRANS*-DEHYDROCROTONIN, A CLERODANE DITERPENE FROM *CROTON CAJUCARA* BENTH. IN MICE FED ON HIGH-FAT DIET

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Introduction And Goals

Croton cajucara Benth. (*Euphorbiaceae*), commonly known as "Sacaca" in many parts of Brazil is a traditional remedy for the treatment of diabetes, hypertension, and high blood cholesterol [5]. Several clerodane diterpenes have been isolated from the bark extracts of this plant which include *trans*-dehydrocrotonin (*t*-DCTN), *trans*-crotonin (*t*-CTN), *cis*-cajucarina B, cajucarina A, and sacacarina [11]. In recent years, the bioactivity of the principal constituent, *t*-DCTN has been extensively studied and was found to possess anti-inflammatory and anti-nociceptive [4], anti-ulcer [3], anti-tumour [9], and anti-oestrogenic [10] properties. Further investigations confirmed that *t*-DCTN is neither genotoxic nor cytotoxic to mouse bone marrow cells [1]. Our previous studies have demonstrated significant hypolipaemic activity in the crude ethanolic extract of sacaca in rats fed a high-fat diet [6] and the hypoglycemic effect of *t*-DCTN in alloxan-induced diabetic rats [7].The present study extends our previous investigation to verify whether *t*-DCTN, the major diterpene present in *Croton cajucara* bark can exert hypolipidaemic effect in mice fed on high-fat diet.

Methodology

- **Isolation of t-DCTN**

t-DCTN was extracted and isolated as described previously [11] from the bark of *Croton cajucara* Benth. collected from Jacunda-PA (Amazón region, Brazil) after its authentication by Dr. Nelson A. Rosa, Museum Paraense Emilio Goeldi (voucher specimen No. 247). The isolated material was characterized by spectroscopy (IR, UV, MS and ^{1}H- and ^{13}C-NMR) as recently described [11].

- **Treatment of animals**

Thirty two male mice were equally divided at random into four groups. The first group of mice served as normal controls and received normal pelletted feed and water *ad libitum*. The mice in the second, third and fourth groups received in addition to pelletted diet and water *ad libitum*, a hyperlipidaemic diet which was a combination of Sunflower oil (10 mL/Kg), 5% cholesterol and 0.5% cholic acid daily by oral gavage [2] for two weeks. *t*-DCTN was administered once daily to mice that belong to groups three and four at oral doses of 25 and 50 mg/Kg, respectively 4 h after feeding. The body weights of animals were recorded initially, and also at the end of the second week. *t*-DCTN was dissolved in dimethylsulfoxide and further dilutions were made in distilled water. The final concentration of the solvent did not exceed 5%. Control animals (second group) received the same volume of vehicle that contained no *t*-DCTN.

- **Analysis of serum lipids**

At the end of two weeks, following an overnight fast, blood samples from the periorbital sinus were collected under ether anaesthesia in ice chilled tubes, and allowed to clot before serum was obtained by subsequent centrifugation at 2500 g for 20 min. The separated serum was stored at –20^{0}C before analysis of lipids. Serum total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-c) and triglyceride (TG) were analysed by the colorimetric method in a semi-automatic analyser (RA-50, Bayer, Brazil) using diagnostic kits (Labtest, Brazil). The serum low density lipoprotein cholesterol (LDL-c) concentration was calculated using the Friedwald formula [8], where LDL-c = TC – (HDL-c + VLDL-c) and VLDL-c = TG/5. The atherogenic index (AI) was expressed as LDL-c + VLDL-c/ HDL-c.

Results

- **Serum lipids**

The serum lipids profiles of mice after a 2-week normal or high-fat diet are shown in Table 1. The normal group of mice fed on normal chow diet had serum total cholesterol and triglycerides levels of 122.21±10.35 and 82.17± 4.80 mg/dL, respectively as against 197.75±1.86 and 342.48±13.76 mg/dL, respectively, in mice on the high-fat diet. *t*-DCTN significantly ($p<0.05$) decreased the high-fat-diet-induced increases in total cholesterol and triglyceride levels, respectively, by 20 and 30% at a dose of 25 mg/Kg and by 50 and 51% at a dose of 50 mg/Kg. The cholesterol fractions, HDL, LDL and VLDL were significantly increased by a high-fat diet. *t*-DCTN had a significant inhibitory effect on these atherogenic lipoproteins, more so at a dose of 50 mg/Kg. Although the level of HDL-c tended to decrease at this dose, the serum HDL-c level remained similar to that of control rats. *t*-DCTN significantly lowered the atherogenic index at both doses employed.

Table 1. Serum lipids in mice fed on normal on high-fat diet and the effects of *t*-DCTN treatment.

Lipid parameter	High-fat-fed and treated		
	Vehicle control	*t*-DCTN 25 mg kg^{-1}/day	*t*-DCTN 50 mg kg^{1}/day
Total choleste ($mg\ dL^{-1}$)	197.75± 12.86*	158.94± 7.64†	120.87± 6.40†
Triglyceride ($mg\ dL^{-1}$)	342.48± 13.76*	171.33± 18.16†	166.80± 13.16†
HDL-c ($mg\ dL^{-1}$)	58.38± 1.75*	61.40± 4.96	47.24± 1.56†
VLDL-c ($mg\ dL^{-1}$)	68.50± 2.75*	34.26± 3.63†	33.34± 2.63†
LDL-c ($mg\ dL^{-1}$)	72.53± 9.13*	70.36± 7.68	43.50± 4.38†
Atherogenic index (AI)	2.38± 0.18*	1.50± 0.14†	1.52± 0.10†

*$P<0.05$, significantly different from normal diet-fed control value. †$P<0.05$, significantly different from vehicle control value.

- **Body weights**

The initial mean body weights of control group of mice fed on normal diet and that of the high-fat diet group of mice that received vehicle were in the order of 30.50 ± 0.50 g and 25.75 ± 0.56 g, respectively. At the end of the experimental period, an increase of 30% in mean body weights was observed in high fat-fed control group of animals. This increase in mean body weight was not observed in the groups of animals treated with 25 or 50 mg kg^{-1} of *t*-DCTN.

Conclusion

In conclusion, the data indicate that orally administered *t*-DCTN is effective in suppressing high-fat-diet-induced hyperlipidaemia in mice and suggest its likely beneficial use as anti-atherogenic agent.

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BI-07 ANTINOCICEPTIVE ACTIVITY OF CRUDE EXTRACTS FROM *CROTON CAJUCARA* LEAVES IN MICE

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Introduction And Goals

Croton cajucara Benth. (*Euphorbiaceae*), commonly known as "sacaca" which grows in Amazon region of Brazil is a traditional medicinal plant and its leaves and stem bark are used in the form of tea or as pills for the treatment of diabetes, high cholesterol and in hepatic and gastrointestinal disturbances [1]. Phytochemical analysis on bark extracts revealed the presence of several terpenoids such as *t*-crotonin, *t*-dehydrocrotonin, cajucarin-β and cajucarinolide (nor-clerodane diterpenes), sacacarin (furano-diterpene) and acetyl aleuritolic acid (triterpene) [2]. *t*-dehydrocrotonin, the major component of bark extracts has been studied extensively for its pharmacological activity and it was found to possess anti-inflammatory and analgesic, anti-ulcer, anti-oestrogen, hypoglycemic and hypolipidemic effects [3, 4]. Although leaf preparations are also much employed by the local people to treat similar disease conditions such as with the bark, so far no published reports on the pharmacological activity are available and in this work we verified a possible antinociceptive activity in the leaf extracts of *C cajucara* using chemical and thermal models of nociception.

Materials And Methods

- **Plant material and extraction**. *Croton cajucara* leaves were collected in April 1994 from Bragança city area, Pará State (Amazonian region, Brazil) and identified by Dr. Nelson A. Rosa. The leaf extracts were obtained by percolation method successively treating with solvents, hexane, chloroform and methanol at room temperature. After filtration, the respective extracts were concentrated until residue in the rotavapor, at 40°C, to get the yield of 14.6, 28.2 and 33.5g, respectively.
- **Animals.** Male Swiss mice weighing 20-25g were used in the study. They were housed at 22°±2°C under a 12h light/ 12h dark cycle. All mice were fed with standard diet (purina chow) and had free access to food and water *ad libitum.* Animals were starved overnight for experimentation.

- **Acetic acid-induced abdominal constriction (writhing movement) test.** Groups of mice received oral doses of test extracts (100 and 200 mg/kg), vehicle (5% gum Arabic, 10 mL/kg in water) or indomethacin (5 mg/kg), 45 min prior to intraperitoneal injection of 0.6% acetic acid (10 mL/kg). The number of writhing movements of each mouse was counted for 20 min, commencing 10 min after injection of acetic acid.

- **Formalin test.** Groups of mice treated as above were injected with 20 μL of 1% formalin (in 0.9% saline, subplantar) and the duration of paw licking was determined 0-5 min (first phase) and 20-25 min (second phase) after formalin injection. Morphine (10 mg/kg. s.c.) pretreated animals were included in the study as positive controls.

- **Hote-plate test.** Mice were pre-selected on the hot-plate at 55±0,5°C, any showing a reaction time (latency for licking the injected paw or jumping in seconds) greater than 10 s were discarded. The mice were then treated with vehicle, leaf extracts (100 and 200 mg/kg, p.o.) or morphine (10 mg/kg, s.c.). The reaction time (s) for each mouse was determined on the hot-plate before and after drug administration at intervals or 30 min for a total period of 90 min. To avoid possible injury, a cut-off period of 45 s was followed while measuring the reaction time.

Results

Table 1. Effect of *Croton cajucara* leaf extracts on acetic acid-induced nociception in mice.

Group	Dose (mg/kg)	Nº of writhing movements (mean^n±s.e.)	% inhibition
Control	----	51.83±5.74	----
Hex. ext.	100	30.50±4.55**	58
	200	24.16±4.64**	47
Chlorof. ext.	100	24.60±6.97**	53
	200	32.17±3.26**	38
Meth. ext.	100	30.37±2.58**	41
	200	29.92±4.33**	42
Indo	5	8.60±4.16***	83

**$p<0.01$; ***$p<0{,}001$ n=6

Table 2. Effect of *Croton cajucara* leaf extracts on formalin-induced nociception in mice.

Group	Dose (mg/kg,)	Paw licking (s) (mean^n±s.e.)	
		1ª phase	2ª phase
Control	----	96.33±7.59	23.67±9.60
Hex. ext.	100	80.16±11.33	1.17±1.17*
	200	82.17±12.63	4.17±2.74*
Chlorof. ext.	100	77.47±11.80	1.27±0.69**
	200	86.26±7.79	3.47±0.84**
Meth. ext.	100	67.57±7.77	9.60±4.95*
	200	76.00±8.32	5.26±4.80*
Indo	5	15.59±2.06***	2.58±1.50**

*$p<0.05$, **$p<0.01$, ***$p<0.001$ n=6

Discussion And Conclusion

The results are shown in tables 1 and 2. As can be observed, all the three extracts (hexanic, chloroformic and methanolic) orally administered (100 and 200 mg/kg), significantly reduced the number of writhing induced by acetic acid in mice in relation to controls that received the vehicle in a manner similar to indomethacin, a known analgesic and anti-inflammatory agent. In formalin test, all the extracts demonstrated antinociceptive activity producing effective blockade of the second phase (20-25 min) nociception but not of the first phase (0-5 min). The formalin test is believed to represent a more valid model of clinical pain [8]. In this test, the first phase response is thought to result from direct chemical activation of myelenated and unmyelenated nociceptive afferent fibres and the second phase response is considered as a consequence of noxious stimulus-evoked long term changes in the properties of spinal dorsal horn neurons [9]. It implies that *C. cajucara* extracts produce a modulatory influence of the functioning of these neurons at the spinal level and exert an antinociceptive effect. However, all the extracts failed to demonstrate any significant influence on hot-plate reaction latency (data not shown) indicating lack of efficacy in suppressing supraspinal nociception. In contrast, morphine, a well known opioid agonist produced a profound antinociceptive effect in both formalin and hot-plate tests.

These results indicate that the antinociceptive activity of the plant is due to both polar and nonpolar compounds present in it. Phytochemical analysis on these extracts revealed the presence of flavonoids (3,7,4´-tri-O-methylkaempferol and 3,7-di-O-methylkaempferol), and steroids (β-sitosterol and stigmasterol) [10] and several of these compounds have been shown to exert analgesic effect in animal models of nociception [11, 12]. In conclusion, the present study evidences the antinociceptive effect of *C. cajucara* leaves and supports their traditional use in folk medicine.

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BI-08 SOME OBSERVATIONS ON THE GASTROINTESTINAL EFFECTS OF EUCALYPTOL (1.8-CINEOLE)

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Introduction

1.8-Cineole (cineole), also known as eucalyptol or cajeputol, is a terpenoid oxide found in many plant essential oils. It is often used as a flavoring agent for food products [2]. Traditionally, it has been used for treating symptoms of airway diseases exacerbated by infection, and a growth inhibitory effect of eucalyptol on several microorganisms has recently been documented [7]. It is also frequently employed by the pharmaceutical industry in drug formulation as a percutaneous enhancer and for its decongestant and antitussive effects and in aromatherapy as a skin stimulant in the form of skin baths [4,5,6,14]. Recent studies have shown its inhibitory effect on the production of inflammatory mediators such as cytokines, prostaglandins, and leukotrienes *in vitro* and *in vivo* [3,11]. In this work, we provided evidence to show that eucalyptol possesses anti-inflammatory and analgesic properties [10]. Due to wide use of eucalyptol in drug formulations, the present study aims to verify its gastrointestinal effects in laboratory animals.

Methodology

- **Animals**

Male Wistar rats (180-200g) and Male Swiss albino mice were used in the study. They were housed in polypropylene cages and maintained at 21 ± 2 ^{o}C with a 12-h light-dark cycle and allowed free access to food (purina chow) and water. Food was withheld for a period of 24 h before the experiments.

- **Ethanol-induced gastric mucosal damage**

Gastric mucosal damage was induced in rats by intragastric instillation of 1.0 ml/rat of absolute ethanol (99.5%) as previously described [8]. The eucalyptol was emulsified in Tween 80 (3% in distilled water) and administered at oral doses of 25, 50, 100 and 200 mg/kg for 45 min before ethanol. Nordihydroguairetic acid (NDGA, 75 mg/kg, p.o.) was used as reference drug for comparison. Control rats received only the vehicle (3% Tween 80). The animals were killed 60 min after ethanol and their stomachs were removed and opened to observe the lesions macroscopically. Lesion severity was determined as previously described [9] and expressed in percentage of the total area of glandular stomach. In a few experiments, rats were simultaneously treated with 8-phenyltheophylline (8-PT, 8 mg/kg, i.p.) or L-NAME (20 mg/kg, i.p.) to verify a possible influence of endogenous adenosine or nitric oxide on eucalyptol effect. The ethanol-induced changes on gastric mucus was indirectly analyzed by determining the amount of Alcian blue bound to the gastric wall [1]. The effect of eucalyptol on gastric secretion was analysed in 4h-pylorus-ligated rats as previously described [12] and the gastric mucosal nonprotein sulfhydryls (NP-SH) were removed according to the method described earlier [13].

- **Small intestinal transit**

The effect of eucalyptol on small intestinal transit was studied in mice. They were treated orally with vehicle (control), eucalyptol (100, 200 and 400 mg/kg) and 1 h later, the mice were given 0.2 ml of a charcoal marker (10% charcoal in 5% gum saline) by oral gastric tube. All the animals were sacrificed after 20 min, the length of small intestine traversed by charcoal marker in relation to total length of small intestine was noted and expressed in percentage. In few experiments, mice were simultaneously treated with 8-phenyltheophylline (8 mg/kg, i.p.) or naloxone (2 mg/kg, s.c.) to verify a possible influence of endogenous adenosine or opioid receptor on eucalyptol effect.

- **Cholera toxin-induced intestinal fluid secretion**

Intestinal secretion was measured *in vivo*, in isolated ligated loops of rat ileum. Rats were pretreated with vehicle (control), eucalyptol (400 mg/kg, p.o.) or 8-phenyltheophylline (8 mg/kg, i.p.) + eucalyptol (400 mg/kg, p.o.). Forty five minutes later, the animals were anaesthetized with pentobarbital sodium (40 mg/kg, i.p.) and laparatomized. Intestinal secretion was induced in a 5 cm long loop of ileum by injecting 0.2 ml of cholera toxin (10 μg in 50 nM Tris buffer). After 4 hours of exposure to cholera toxin, the animals were sacrificed intestinal loops were collected and weighed. The intestinal secretion was expressed in terms of mg/cm of changes. A group that received only Tris buffer was included in the study.

Results

- **Ethanol-induced gastric mucosal damage**

Gastric Lesions. The lesion area in control rats was 16.91 ± 2.56 mm^{2}. Eucalyptol at doses of 25, 50, 100 and 200 mg/kg prevented the gastric mucosal injury by 21, 74, 90 and 92%, respectively. NDGA (75 mg/kg) also prevented the mucosal damage by 84%. The gastroprotective effect of eucalyptol (200 mg/kg) against ethanol injury was not significantly altered in rats treated with 8-PT or L-NAME.

- **Gastric Wall Mucus and Mucosal Sulfhydryls**

The content of gastric mucus was slightly, but not significantly, elevated in the ethanol-treated animals (53.91 ± 7.05 μg/g) compared to normal rats (31.39 ±5.42 μg/g). Eucalyptol at 50 mg/kg significantly enhanced the gastric mucus content (91.06 ± 16.56 μg/g), but at higher doses it showed no significant effect. Treatment of animals with absolute ethanol significantly lowered the gastric NP-SH concentration (187.66 ± 16.46 μmol/g) by 37% compared to vehicle-treated rats (296.77 ± 16.46 μmol/g). The treatment with absolute ethanol + eucalyptol (50, 100 and 200 mg/kg) showed lesser reductions (31, 22 and 5%, respectively) than the group treated with ethanol alone.

- **Gastric Secretion**

The gastric secretory volume and total acid output in rats that received the vehicle were 1.80 ± 0.09 ml/h and 229.92 ± 20.17 μeq/h, respectively. Eucalyptol (50 mg/kg) caused a slight increase in the above parameters; higher doses (100 and 200 mg/kg) significantly reduced the gastric secretory volume (41 and 52%) as well as the total acid output (40 and 84%), respectively.

- **Small intestinal transit**

The percentage of small intestine traversed by charcoal marker in group control was 59.89±2.19 %. Eucalyptol (400 mg/kg) significantly reduced the intestinal transit (5.19±3.43 %), but at lower doses it showed no significant effect. Naloxone (2 mg/kg) effectively blocked the inhibitory effect of eucalyptol (400 mg/kg). The inhibitory effect of eucalyptol (400 mg/kg) was not altered in rats treated with 8-PT.

- **Cholera toxin-induced intestinal fluid secretion**

Cholera toxin in control rats caused a significant increase of intestinal fluid accumulation (250.93 ± 47.53 mg/cm) when compared with rats that received Tris buffer alone (174.21 ± 19.59 mg/cm). Rats pretreated with eucalyptol (400 mg/kg) showed significantly less accumulation of intestinal fluid (128.43 ± 10.32 mg/cm) when compared with toxin control. The inhibitory effect of eucalyptol was not altered in rats treated with 8-PT.

Conclusion

Data demonstrate that eucalyptol offers protection against ethanol damage and the gastroprotection, in part may involve its antioxidant action, preventing ethanol-induced depletion of NP-SH, and also, partly due to lipoxygenase inhibition, blocking leucotrienes formation. The gastrointestinal transit is inhibited by eucalyptol and the effect of eucalyptol is antagonized by naloxone but not by 8-PT, which indicates the participation of endogeneous opioids and non participation of endogenous adenosine. Eucalyptol promotes inhibition of the intestinal secretion induced by cholera toxin. This effect was not antagonized by the adenosine antagonist 8-PT, which indicates the non participation of adenosine endogenous in its mechanism. Taken together, these observations with our recent findings on its anti-inflammatory and analgesic potential it can be suggested that eucalyptol may be an ideal candidate for novel therapeutic applications.

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BI-09 THE USE OF INTESTINAL PERFUSION TECHNIQUE IN RATS TO STUDY IRON ABSORPTION KINETICS QUELATE PEPTIDES-IRON

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Introduction

Iron organic deficiency is responsible by iron deficiency anemia, nutritional failure, which affect about 60% of world population. The main difficulties to combat this sickness is bioavailability of intake iron and the factors understanding that govern intestinal absorption of this mineral elements. Although some papers consider that minerals are best absorbed the lower parts of the intestine, the duodenum is consider being the main site of ion iron absorption. Many researches taking into consideration that intestinal absorption can be defined, operationally, as the total disappearance of a substance of intestinal lumen. This way intestinal perfusion technique “in situ” has been used to directly measure drugs absorption tax. This study purpose was to compare iron absorption tax, from quelate peptides-iron along the small intestine using intestinal perfusion “in situ” by recirculation method.

Experimental Method

- **Material**

Rats male, of Wistar specimen, weighting 170-200g. Casein hydrolyzed (BIOBRAS); Piridil-bis ferril triazine sulfonate - PBTS (BIOLAB); pentobarbital sodium (ABBOT), Iron sulfonate (MERCK). Peristaltic two way pump; UV/VIS Spectrophotometer; Atomic Absorption Spectrophotometer (AAS).

- **Quelate peptides-iron preparation and dosage of iron quelate**

The quelate iron-peptides was prepared as described by Chaud, 1999[1]. The iron content in quelate was determined by AAS after acid digestion.

- **Experimental Part**

The groups were made up of 8 animals, all weighting 183±11.5g and hemoglobin rate of 11.4±0.5mg/dL. Deprived of solid meal for 16 hours before test start, the animals, anestesied with pentobarbital sodium, underwent abdominal incision of nearly 10 cm, through which the small intestine was exposed. Each intestinal segment was individually cannulated with polyethylene cannula, fixed with suture thread avoiding obstructing important sanguineous vessels. The corporeal temperature was controlled and kept with the help of infrared lamp. The aqueous dispersion of peptides-iron quelate or ferrous sulfate in the 0.5 and 1.0mg Fe/mL concentration were continuously perfused in the isolated intestinal segment, thorough recirculation procedure, for 40 minutes, in the 0.30mL-0.40mL/minute velocity. The system of perfusion is represented schematically in Figure 1. The blood was collected for cardiac punction immediately after perfusion procedure, and serum iron determined for colorimetric method after PBTS reaction. The serum iron basal (control) was dosed in two groups (n=6), one of which submitted to perfusion procedure with NaCl 0,9% solution. The aqueous dispersion perfused was kept at 37° C without light and with constant agitation.

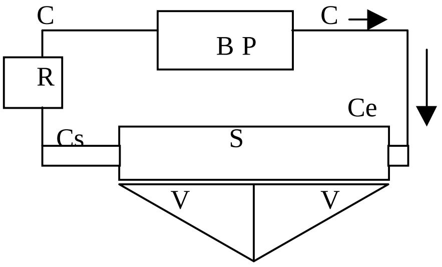

Figure 1: Schematic representation of recirculation perfusion technique.
R: reservatore, PB: peristaltic pump; C: cannula, Ce, Cx: respectively, entrance and exit cannula, S: intestinal segment perfused, V: sanguineous vessels.

Results and Discussion

The iron concentration determined by AAE in the peptides-iron quelate, after drying by lyophilization, was 9.45g Fe/100g of quelate. The hemoglobin dosage (11.4±0.5mg/dL) denotes iron repletion. The Table 1 shows iron serum in the control groups (basal iron). The Table 2 shows the results of serum iron dosage after perfusion aqueous dispersion of iron sulfate or peptides- iron quelate. The results are graphically shown in the Figure 2. The results were statistically valued for Variance Analyses (ANOVA) and Dunnett Test.

Table 1
Serum iron in the control groups (n=6)

Control groups	Serum iron µg/100mL
Without Perfusion	120 ± 6.72
Saline Perfusion	118 ± 9.86

Table 2
Iron serum after perfusion of $FeSO_4$ or iron-peptides in each intestinal segment.

Iron serum µg/100mL				
	$FeSO_4$ (mg/mL)		Fe-peptides (mg/mL)	
	0.5	1.0	0.5	1.0
Duodenum (n=6)	203.6± 10.4	236.1± 10.9	323.2 ±15.9	492.6± 9.5
Jejun.prox. (n=7)	120.5± 12.34	158.4± 19.1	312.0 ±13.6	393.4± 18.52
Jejun.distal (n=7	117.9± 5.52	129.9± 10.9	258.1 ±17.6	362.5± 18.50

The number among parenthesis represents the animals utilized in each intestinal segment perfused. Jejun.prox. is Jejunum proximal

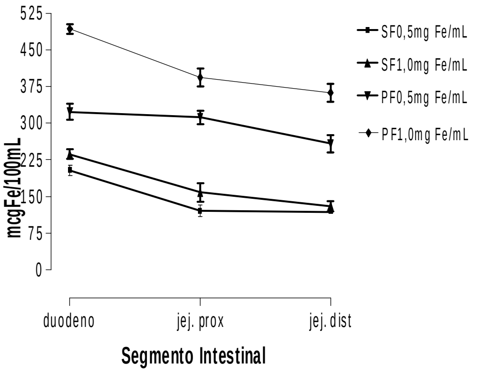

Figure 2 – Iron absorption in µg/mL after intestinal perfusion of iron sulfate (SF), or iron-peptides (PF) in aqueous dispersion in the 0.5 or 1.0 mg Fe/mL concentration. The vertical bar corresponds to means standard deviation.

The iron absorbed rate, when $FeSO_4$ solution was perfused shows that in the 0.5mg Fe/mL concentration the iron is absorbed only by the duodenum. The absorption rate obtained as of proximal and distal jejune perfusion are similar statistically in the control groups ($p > 0.05$). When the concentration of perfused solution was 1mgFe/mL only the absorption thorough distal jejune didn't present statistical difference when compared to control groups, these results show that iron ion is better absorbed by the duodenum and that the absorption thorough on iron concentration availability for absorption. The groups treated with peptide-iron quelate show higher rates of iron when compared to animals treated with $FeSO_4$, independent on iron concentration perfused and from the perfusion place ($p<0.05$). The difference in the iron absorption was statistically significant among groups treated with peptides-iron ($p<0.05$) in the 0.5 mg Fe/mL and 1.0mgFe/mL concentrations. The groups treated with more concentrated dispersion showed higher absorption. In the groups treated with 1.0mgFe/mL the difference in the iron absorption was significant only between groups duodenum and distal jejunum ($p<0.05$). For the groups treated with 1.0mgFe/mL, the iron rate absorbed in the duodenum was higher than those absorbed in the proximal and distal jejunum. The mechanism by which the absorption of iron-peptides quelate happens cannot be clarified. Probably the quelate depend less of transport mechanisms by intestinal membrane.

Conclusion

- The peptides-iron quelate furthers better iron absorption indices than $FeSO_4$.
- Iron absorption from quelate peptides-iron happens a long all small intestine.
- Independently of the iron source the duodenum is a better absorption site of this mineral element.

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BI-10 ANALGESIC ACTIVITY OF ESSENTIAL OIL OF *OCIMUM MICRANTHUM* WILLD FROM NORTHEASTERN BRAZIL

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Introduction

Plants of genus *Ocimum* are widely distributed in tropical and warm temperate regions. The essential oils of *Ocimum* species are used either in perfumery or pharmaceutical industries. *Ocimum micranthum,* Willd, a strongly aromatic annual herb native to the lowlands of Central and South America and West Indies, is used to flavor beverages and soups. It is used in domestic medicine for treating colds, fever, stomach disturbances, and dysentery and as a remedy for screwworms parasitizing nasal passages of people in the tropics. A decoction of the plant is also used to kill the larvae. The plant is locally used in the treatment of epilepsy, nervous trouble, earaches, as a remedy for influenza, colic, and convulsion in children, and for painful menstruation. Although not well known but yet highly aromatic plants such as *O. micranthum* could be sources of specific natural products or used in interspecies hybridizations [6]. The essential oil content of plant parts shows a great variability (with 1.54, 0.63, and 0.08, percent volume/weight, in the leaves, flowers, and stems, respectively). Twenty compounds were identified in the essential oil: 1,8-cineole, eugenol, β-caryophyllene, β- selinene, and elemene isomers are the major constituents. Essential oil composition also varied according to plant part. Eugenol, the major constituent in leaves, was present only in trace amounts in flowers and stems. β-Selinene was a major constituent in the flowers and stems. Total sesquiterpenes accounted for 48.4, 85.8, and 78.5% of the essential oil in the leaves, flowers and stems, respectively [1]. Chemical composition of the leaf oil of *O. micranthum* collected in North of Brazil and in India has been subjected to previous study. The major components reported were β- elemene, β-caryophyllene and Isoeugenol in the Brazilian specimens), whereas eugenol, 1,8-cineole, β-caryophyllene and γ- elemene were reported in specimens collected in India. Anticonvulsant and antispasmodic activities of *O. micranthum* have also been reported [7].

Objective

The main objective of the present paper was to study the analgesic effect of the essential oil (EO) from the leaves of *O. micranthum,* in order to elucidate its mechanisms of action.

Materials and methods

Fresh leaves of *O. micranthum* Willd (*O. campechanum*) were collected at the Medicine Plants Garden of the Federal University of Ceará (Brazil). The exsicatae is deposited at the Prisco Bezerra Herbarium under the number 29315. Steam distillation of the fresh leaves produced the essential oil (UFC code- F2176) with a 3.05% yield.

- **Writhing test.** Male Swiss mice (25-30g) were used. The animals (6-12 per group) were pretreated with the (1, 5, 10 mg/kg, i.p or p.o) or vehicle (distilled water: 0.1ml/10g, ip or p.o). Thirty minutes later, they were injected with acetic acid 0.6% according to the method of Koster et al. [4]. Then, after 10 min, the number of writhing/20min was registered. The antinociceptive activity was evaluated in terms of the percentage of contraction inhibitions. In some experiments, naloxone (4mg/kg, sc), an opioid antagonist, was administered 15min before the treatment with the EO.
- **Formalin test.** The formalin test was based on the method of Dubuisson and Dennis modified by Hunskaar and Hole [2]. Male Swiss mice, (6-12 per group), 25-30g, were pretreated with OE (0.1, 1, 2.5, 5mg/kg, p.o or i.p), morphine (4mg/kg, sc) or vehicle (0.1ml distilled water/10g, p.o or i.p). One hour (p.o) or 30min (i.p) later, the animals were injected in the right hind paw with 20μL formalin 1%. Then, the animals were observed for the pain reaction (licking time) during the first phase (0-5min), neurogenic, and the second phase (20-25min), inflammatory. In some experiments, mice were pretreated with naloxone (4mg/kg, sc) and, after 15min animals received the EO (5mg/kg p.o or i.p), morphine (4mg/kg sc) or vehicle; 1h or 30min later, they were then injected with formalin. In another group, mice were pretreated with L-arginine (300, 600mg/kg, i.p)

and, after 5min, the animals received L-NAME (18.7, 37.5mg/kg, i.p) and EO (5mg/kg, i.p); 30min later, they were injected with formalin and observed during the first and second phase of the formalin response.

- **Hot-plate.** Animals treated with EO (2.5, 5, 10, 100, 200, i.p) were submitted to the hot-plate test (55±1°C) followed by measurements of reaction latencies at 30, 60, 90 and 120 sec.
- **Statistical analysis**. Data were analyzed by Analysis of Variance (ANOVA) followed by Student-Newman-Keuls *post-hoc* test when necessary. Differences bellow the probability level of 0.05 were considered statistically significant.

Results and discussions

Acetic acid-induced abdominal constrictions demonstrated that EO (1.0, 5.0 e 10mg/kg, ip), inhibited the acetic acid contractions by 71 (10.5±4.2), 79 (8.0±1.8) and 73% (9.7±2.5) as compared with control (36.0±2.7 contractions per 20min). Naloxone did not reverse the antinociceptive effect, suggesting the non-involvement of the opioid system in this effect.

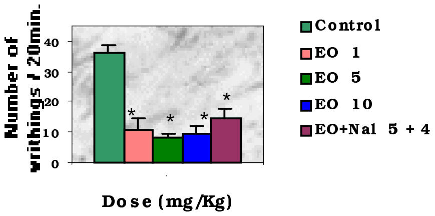

Figure 1. Influence of naloxone pretreatment on the antinoceptive effect of essential oil of *O. micranthum* on acetic acid test. *p<0.05 compared to control group (ANOVA followed by Student-Newman-Keuls test).

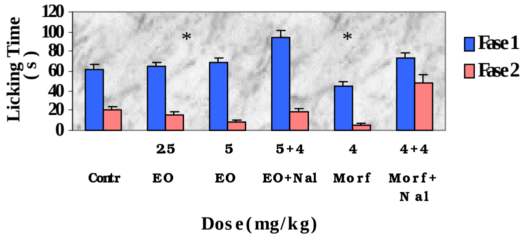

Figure 2. Influence of naloxone pretreatment on the antinociceptive effect of essential oil of *O. micranthum* on the formalin test. *p<0.05 compared to control group (ANOVA followed by Student-Newman-Keuls test).

The results of the formalin test, was also used to study the antinociceptive effect. The EO at doses of 2.5, 5 and morphine 4 mg/Kg, sc, caused inhibition of 52 (13.6 ± 3.1), 61(8. ± 1.5), and 89% (2.18±2.04s) of the second phase, respectively. Again, the pretreatment with naloxone did not reverse the antinociception observed after the administration of EO. However, naloxone reversed as expected the antinociceptive effect demonstrated with morphine used as standard. In order to verify the possible involvement of the nitric oxide system in the antinociception produced by the essential oil, its effect was studied in the presence of L-arginine, known to block the nociception observed with L-NAME. L-arginine (600mg/kg,i.p) antagonized the EO effect on the second phase of the formalin test The blockade was complete in the group administered with the EO and the higher dose of L-arginine, 600mg/kg,i.p. In the hot-plate it was demonstrated that all doses of the EO (2.5, 5.0, 10, 100 and 200mg/kg, i.p) failed to modify the reaction time of mice.

Conclusions

The antinociceptive effect of the EO was not blocked by the opioid antagonist naloxone, suggesting the non-involvement of the opioid system. The mechanism of action is not clear but could be related to the nitric oxide system (NO). The present work showed that L-arginine (substrate for the synthesis of nitric oxide) in mice (600mg/kg,ip) completely reversed the antinociception in the second phase. L-NAME (which inhibits the nitric oxide synthesis) produced a significant and predominant decrease of the nociception in the second phase. Similarly, the effect of the EO (5mg/kg,ip) was also antagonized by L-arginine in the second phase, pointing out to the role of nitric oxide system in the observed analgesic effect of the essential oil of *O. micranthum.*

Acknowledments

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BI-11 ACTION OF THYROXIN ADMINISTERED TO FEMALE RATS BEFORE COPULATION ON THE PROGENY

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Introduction

The hormones are essential elements to organisms. The thyroid hormones are fundamental to metabolism [1], development of nervous system, organic growth [2] and they are produced in more quantity during pregnancy, which promotes the metabolic increase of other circulating hormones [3].

The fetal thyroid functions, by the end of pregnancy, with autonomy in many mammalian species, including men and rats, but the maternal thyroid hormones also have participation on the fetal economy.

The hypothyroidism and the hyperthyroidism appear in abnormal states. When the hyperthyroidism, in female, occurs before copulation, alterations on progeny are provoked [3].

Due to this alteration, we decided to analyze the hormonal modification in the newborn rats.

Methodology

Female rats (Wistar), weighing between 220-270g, were divided in 2 groups: the control group received saline solution (0.9%); the treated group received thyroxin-T_4 (2µg/100g of body weight/day) for 2 days, before the copulation, by intraperitoneal way. The progeny was sacrificed aging 10, 20, 30 and 60 days for blood collecting and serum attainment. The hormonal dosages of total T_4 and T_3 were measured by RIA. Glucose was evaluated by colorimetry.

Results and discussion

The Fig.1 shows the effect, on progeny, of the treatment with thyroxin before copulation in relation to the serum levels of total T_4. It is observed that, at 20 days after birth, the descendant presented a reduction, statistically significant, of 85.7% when compared to the control group.

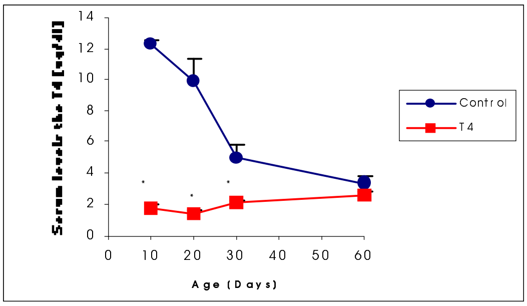

Figura 1 – Serum levels of descendant's T4 (µg/dl) of female rats treated with thyroxin before copulation, n=10 and *p≤0.05.

The Figure 2 illustrate the effects of the treatment on serum levels of T_3, the progeny (also aging 20 days) of the treated group presented a reduction, statistically significant, of 66.7%.

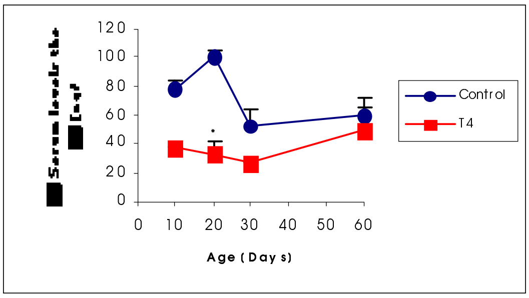

Figure 2 – Serum levels of descendant's T_3 (nmol/dl)of female rats treated with thyroxin before copulation, n=10 and *p<0.05.

It can be observed that on Figure 3, the serum levels of glucose of the group treated with thyroxin before copulation, at 20 days after birth, presented a significant reduction of 77.9%, in relation to the control group.

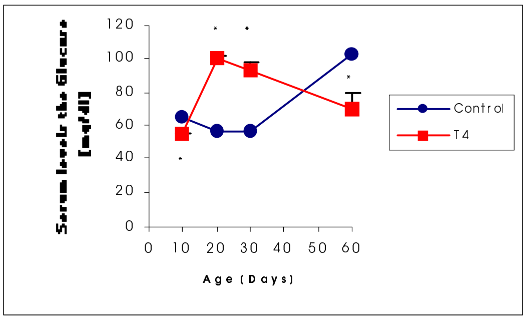

Figure 3 – Serum levels of descendant’s glucose(mg/dl) of female rats treated with thyroxin before copulation , n=10 and *p≤0.05.

These results show that the administration of thyroxin to female rats before copulation causes modifications on the serum levels of T_4, T_3 and glucose of the progeny which suggests that the maternal thyroxin may contribute to the activation of 5’deiodinase enzyme, responsible for the conversion process of T_4 to T_3.

Conclusion

It is concluded that the thyroxin administration, to female rats before copulation, affects the fetal thyroid gland function, which causes alterations on the serum levels of T_4, T_3 and glucose of the progeny after birth, indicating that the maternal hyperthyroidism influences the post-natal life of the rat.

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BI-12 BUSPIRONE EFFECTS ON ELEVATED PLUS MAZE MODEL ON RAT BEHAVIOR

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Abstract

This work studied the effects of buspirone (Busp) on behavior of rats in the elevated plus maze. Busp (5 or 10 mg/kg, p.o.) was administered a twice daily to female Wistar rats during 7 days. After 1 h the last administration of drug, animals were submitted to behavioral test. Busp 5 but not 10 mg/kg increased all parameters indicative of anxiety on plus maze, suggesting an anxiolytic profile. The absence of effects in the higher dose, probably occurred due of great decrease of the locomotor activity (data not shown) affecting the conventional measures of anxiety on the plus maze.

Introduction

Buspirone, a 5-HT1A a partial agonist [1] is an anxiolytic drug, as potent as the benzodiazepines without produce the sedation or motor impairment found with these drugs [2].

The elevated plus maze has been also used for detecting both anxiolytic and anxiogenic effects of drugs in rats and mice [3].

The objective of this work was verify the behavior of rats in this experimental model.

Experimental method

- **Animal**
  Female Wistar rats (150-200 g) were used.

- **Drug**
  Buspirone hydrochloride (BuSpar, Bristol-Meyer Squibb Brazil).

- **Treatment**
  Animals were treated twice daily for 7 days with buspirone (5 and 10 mg/kg, p.o.). Controls received the same volume of saline. 60 min after the last administration, animals were submitted to behavioral testing

Behavior on the Elevated Plus Maze

The elevated plus maze was made of wood, according to specifications described elsewhere [4].

- **Procedure**

The rats were placed individually, 1 h after the last administration, in the central square facing a closed arms and allowed to explore the elevated plus maze for 5 min. The conventional measures (number of entries into open and closed arms, and the time spent on open and closed arms) were recorded. The behavior of the animals was recorded by an observer

- **Statistical Analysis**

  Each animal, the total number of entries (open + closed arms), the percentage of open arms entries (100 x open/total), and the time spent in the open arms were calculated using ANOVA for multiple comparisons and Dunnett's as post *hoc test* $p<0.05$.

Results and discussion

Table 1 and 2 show the effect of buspirone on conventional anxiety indexes in elevated plus maze. There were a significant increase around 180% in all parameters observed: number of entries into the open-arms (NEOA), percentage of open-arms entries (PEOA), the time spent in open arms (TSOA) and percentage of time spent on open arms (PTOA) for busp (5 mg/kg), but not for busp 10 mg/kg when compared to saline controls

Table 1- Effect of busp (5 or 10 mg/kg, p.o.) or saline (control) twice a day for 7 days in the elevated plus maze on the number of entries into the open-arms (NEOA) and on the percentage of open-arms entries (PEOA).

Groups	NEOA	PEOA
Control	0.58 ± 0.23	6.65 ± 2.41
Busp 5	2.25 ± 0.35*	21.6 ± 3.06 *
Busp 10	0.93 ± 0.25	14.65 ± 4.03

Values are expressed as means ± SEM, the number of rats in each group varied from 12 to 24. (ANOVA and Dunnet as the *post hoc* test).

The elevated plus maze is based on the inate fear rodents have for open and elevated spaces [5]. Rats on the elevated plus maze tend to avoid the open arms and prefer to stay in the enclosed arms. When confined to open arms, rats show behavioral and physiological manifestation of fear, such as freezing, defecation, and increases in plasma corticosteroids [3, 6].

The traditional anxiety indexes in elevated plus maze show a good sensitivity to both anxiolytic and anxiogenic drug acting at GAGAA/benzodiazepine receptor complex [7]. However, serotonergic drugs in anxiety models, include buspirone, has produced inconsistent results in a wide range of animal models of anxiety. In particular, in the elevated plus maze, busp has been show to have no effect, anxiolytic-like effects and ansiogenic-like effects [8, 9]. In this study all parameters studied (NEOA, PEOA, TSOA e PTOA), increased suggesting an anxiolytic- like effect of busp 5 mg/kg but not 10mg/kg on plus maze. The absence of effects in the higher dose, probably occurred due of great decrease of locomotor activity affecting the conventional measures on the plus maze (data not shown).

Table 2- Effect of busp (5 or 10 mg/kg, p.o.) or saline (control) twice a day for 7 days in the elevated plus maze on the time spent in open arms (TSOA) and percentage of time spent on open arms (PTOA).

Groups	TSOA	PTOA
Control	7.25 ± 3.01	2.73 ± 1.13
Busp 5	29.46 ± 5.63*	11.43 ± 2.11*
Busp 10	15.79 ± 6.05	5.88 ± 2.269

Values are expressed as (mean ± SEM), the number of rats in each group varied from 12 to 24 (ANOVA and Dunnet as the *post hoc* test).

Conclusion

These results suggest an anxiolytic-like effects of busp elevated plus maze models.

Acknowledgments

This work had financial support from the Brazilian National Research Council (CNPq).

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BI-13 EFFECTS OF HALOPERIDOL ON BEHAVIOR AND DENSITY OF DOPAMINERGIC D2-LIKE RECEPTORS IN OLD RATS

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Abstract

The present work shows the effects of a typical neuroleptic drug (haloperidol) on rat behavior (catalepsy and spontaneous locomotor activity) and dopaminergic D2-like receptor densities in the hippocampus and striatum from old rats (12-18 months old). Male Wistar rats were treated daily for 30 days with haloperidol (HAL 1mg/kg, i.p.). At several times, 1 h after the last drug injection, animals were submitted to behavioral tests. Old rats presented catalepsy and no locomotor activity after HAL as compared to controls. Scatchard analysis showed that HAL caused a D2 up regulation in the hippocampus and striatum from old rats. This result suggest the effect behavior was influence of alteration of system dopaminergic.

Introduction

The striatum is believed to be the main brain structure responsible for the catalepsy induced by neuroleptic drugs [1]. Besides, it has been suggested that the dopaminergic mesolimbic system is the critical site of action for their antipsychotic effect [2]. In rodents, spontaneous locomotor activity (SLA) is partially mediated by the dopamine mesolimbic system [3]. On the other hand, the demonstration of a dopaminergic innervation in the hippocampus associated with evidences suggesting its important role in schizophrenia [4] and behavior led us to study possible alterations of D2 receptor density in this brain area after haloperidol treatment. The objectives of the present work were: to compare D2 receptor densities in the hippocampus and striatum from old rats after haloperidol treatment and study the correlation between behavioral effects and receptor densities after the repeated administration of haloperidol.

Experimental methods

Animals- Wistar rats old (12-18 months of age, 300-350 g) were used.

Drug- Haloperidol (Haldol, 5 mg/ml) was diluted in distilled water, and administered intraperitoneally (i.p.) in volumes of 1 ml/kg body weight.

- **Treatment** - Animals (6-9 per group) were treated daily, at 12 a.m., for 30 days with HAL (1 mg/kg, i.p.) and sacrificed 24 after the last injection for binding assays. Before death and 1 h after drug injection, animals were submitted to behavioral studies. Controls received equivalent volume of distilled water.
- **Behavioral studies** - Tests (catalepsy and locomotor activity) were carried out on the 1st, 3rd, 5th, 10th, 15th, 25th and 30th days of treatment, 1 h after drug injection. Catalepsy was assessed according to the method described by Ahlenius and Hillegaart [3]. Catalepsy was considered as positive when the animals sustained the posture for over 60 s. Locomotor activity was performed according to Jorgensen *et al.* [5]. After 1 min of habituation, on a red open field area (50 × 50 cm) the locomotor activity for the following 3 min period was measured and registered as the number of crossings from one quadrant to another.
- **Binding studies** - For the binding assays, the method of Kessler *et al.* [6] was used. Animals were decapitated, and the hippocampus and striatum were dissected under ice for the preparation of the homogenate. The homogenate containing 60-120 µg of protein was incubated in the presence of 10 µM mianserine for blocking serotonergic receptors. Several ligand concentrations [3H]-spiroperidol (109 Ci/mmol, Amersham, USA) ranging from 0.04 to 2.29 nM were used in a final volume of 0.2 ml. The specific binding was calculated by subtracting the total minus nonspecific binding done in the presence of 5 µM dopamine. Data were expressed as fentomoles/per mg of protein. Protein was determined by the method of Lowry *et al.* [7].
- **Statistical analyses** - All results are presented as mean ± S.E.M. Parametric test was used. Data were compared with unpaired Student's *t*-test.

Results and discussion

The result showed the after treatment maximum time on the bar (Figure 1-A) was reached 1h after the 1st haloperidol exposure (1st day), and remained as such up to the 30th day (146.5±2.98 s). Also, these animal showed a complete blockade of locomotor activity (Figure 1-B) during all period as compared to controls (0.08±0.08 and 6.0±0.68

crossings/3min for the haloperidol group and controls, respectively), at the 30th day. Table 1 shows [3H]-spiroperidol binding in the hippocampus and striatum of old rats, 24 h after the last haloperidol administration (1 mg/kg, i.p.) for 30 days. The results show receptor upregulation (25 and 17% increases) in the hippocampus [t(8)=3.117, p<0.0143] and striatum [t(9)=2.388, p<0.0407], respectively, and no alteration in Kd values.

Table 1- Scatchard Analyses Data from [3H]-Spiroperidol Binding in the Hippocampus and Striatum of Old Rats.

Group	**Hppocampus**		**Striatum**	
	Bmax	Kd	Bmax	Kd
C	107.2±5.3 (6)	1.24±0.10 (6)	101.6±6.1 (6)	1.14±0.13 (6)
H	133.8±6.6 (4)*	1.42±0.15 (4)	118.7±2.6 (5)*	0.95±0.08 (5)

Numbers represent means ± SEM of groups of animals (in parentheses) treated daily with haloperidol-H (1mg/kg, i.p.) for 30 days and sacrificed 24h after the last injection. *p<0.05, Student's *t*-test (control vs. haloperidol).

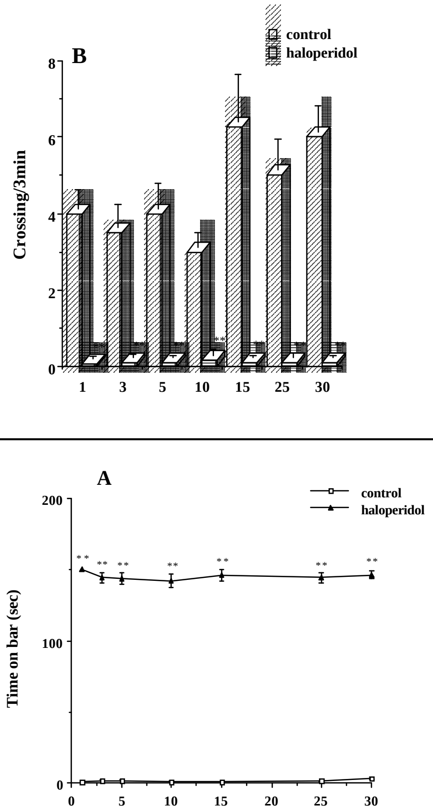

Figure 1- Test of catalepsy (A) and spontaneous locomotor activity (B) in old rats (n=6-14) at the 1st, 3rd, 5th, 10th, 15th, 25th and 30th days of treatment 1h after administration of haloperidol (1mg/kg, i.p.) or distilled water (control). The catalepsy was measured by the time on the grid (s) and spontaneous locomotor activity was measured by the number of crossings from a quadrant to another during a 3 min period. **p<0.01, Student's *t*-test (control vs. haloperidol).

Conclusion

Haloperidol produces alterations in catalepsy and SLA, as well as on binding of D2 receptor in old rats.

Acknowledgment

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BI-14 COCAINE REPEATED ADMINISTRATION AND EARLY WITHDRAWAL DOWNREGULATES MUSCARINIC AND DOPAMINERGIC D$_1$-RECEPTORS IN RAT STRIATUM

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Abstract

The present work studied alterations in muscarinic and dopaminergic D$_1$ receptors density in rat brain *striatum* 24 h after withdrawal from cocaine repeated administration (20 and 30 mg/kg, i.p., daily for 7 days). Binding assays were performed in *striatum* homogenates, and the ligands used were [^{3}H]-N-methylscopolamine, [^{3}H]-SCH 23390, for muscarinic and D$_1$-like receptors, respectively. Scatchard analyses of muscarinic and D$_1$ receptors presented a downregulation with both doses. These findings indicate that changes in brain neurochemistry caused by cocaine remains after a 24 h withdrawal period.

Introduction

Dopaminergic D$_1$, D$_2$ and D$_3$ receptors may be involved in the reinforcing effects of the mesolimbic dopaminergic system. D$_1$ as well as D$_2$ receptors seems to be involved in cocaine addiction; the first, suppressing self-administration and, the later, inducing drug-seeking behavior [1]. Besides dopaminergic system, other neurotransmitters might play a role in cocaine actions. Lipton et al. [2] observed an increase in muscarinic receptors in the hippocampus 12 h after cocaine withdrawal. This increase was no longer evident 48 h later, and decreased 21 days after withdrawal. Similar changes in muscarinic receptors are also apparent in the *striatum.* The objective of the present work was to determine changes remaining after a 24 h withdrawal from cocaine administration in muscarinic and dopaminergic receptors densities in rat *striatum.*

Experimental methods

- **Animals and schedule of drug administration**

Male Wistar rats (180-200 g) were treated daily for 7 days with cocaine (20 and 30 mg/kg, i.p.). Controls received the same volume of saline. Animals were decapitated 24 h after the last injection and *striatum* was used to prepare 10 % homogenates.

- **Drugs**

The drug used was cocaine hydrochloride. Radioactive isotopes were purchased from Amersham Life Science, USA.

- **Statistical analyses**

Data are expressed as mean ± S.E.M. ANOVA followed by Student-Newman-Keuls test were used to identify differences between experimental groups. Values of P< 0.05 were considered significant.

- **Muscarinic and Dopaminergic Receptor Binding Assay**

Muscarinic receptors were measured using [3H]-N-methylscopolamine, ([3H]-NMS, 85 Ci/mmol, New England Nuclear, Boston, MA), according to Dombrowski *et al.* [3], with some modifications. Concentrations of [3H]-NMS ranging from 0.119 to 5.95 nM in a final volume of 0.2 mL were used. Specific binding was calculated as total minus nonspecific binding performed in the presence of atropine (12.5 μM), and results are reported as fentomoles per milligram of protein. Protein was determined using bovine serum albumin as standard [5].

Densities of D$_1$-like receptors were determined according to methods described by Meltzer *et al.*, [4]. The specific ligand [^{3}H]-SCH 23390 (87 Ci/mmol from New England Nuclear) was used. Concentrations of [^{3}H]-SCH 23390 ranging from 0.115 to 9.2 nM in a final volume of 0.2 mL were used. Specific binding was defined as total minus non-specific binding carried out in the presence of 10 μM butaclamol.

Results and discussion

Table 1 presents Bmax and Kd values for M$_1$ plus M$_2$ like receptors. There was a downregulation of 54 and 43.6% in Bmax values with cocaine 20 and 30 mg/kg, respectively, as compared to controls, with no difference in dissociation constant values. Decreases in D$_1$ receptor density of 37.6 and 31.8%, respectively, were seen with cocaine 20 and 30 mg/kg, i.p., with no alterations in dissociation constant values (Table 2).

This work showed a downregulation of D$_1$ receptors after repeated cocaine administration. Similarly, Graziella de Montis *et al.* [6] showed that animals receiving cocaine presented a downregulation of dopamine D_1 receptor number and dopamine stimulated adenylyl cyclase activity in the *nucleus accumbens*, as compared to saline treated rats. Such changes might underlie some of the behavioral consequences, like drug dependence or craving, after a long-term cocaine use, suggesting that D_1 receptors might be good targets for the development of new therapeutic strategies.

Table 1 - Effects of Cocaine on Muscarinic Receptor Density (B_{max}) and K_d Values in Rat *Striatum.*

Cocaine (mg/kg, I.P.)	M_1+M_2	
	B_{max}	K_d
0	358.2 ± 44.5 (5)	1.23 ± 0.24 (5)
20	163.6 ± 11.9 (4)*	1.08 ± 0.41 (4)
30	201.9 ± 28.5 (5)*	1.39 ± 0.17 (5)

Data are reported as means ± SEM for the number of experiments shown in parentheses. For statistical analyses, ANOVA and the Student-Newman-Keuls test as a *post hoc* one were used. *p< 0.05 compared to controls.

Table 2- Effects of Cocaine on D_1 dopaminergic Receptor Density (B_{max}) and kd Values in Rat *Striatum.*

Cocaine (mg/kg, I.P.)	D_1	
	B_{max}	K_d
0	128.0 ± 14.07 (5)	5.78 ± 0.60 (5)
20	79.9 ± 5.04 (6)*	6.28 ± 0.35 (6)
30	87.3 ± 5.40 (5)*	7.11 ± 0.61 (5)

Data are reported as means ± SEM for the number of experiments shown in parentheses. For statistical analyses, ANOVA and the Student-Newman-Keuls test as a *post hoc* one were used. *p< 0.05 compared to controls.

The results of this work also showed that 24 h after the last injection there was a downregulation of muscarinic receptors in rat *striatum*. This is not in agreement with Sousa *et al.* [7], who observed an upregulation of muscarinic receptors 24 h after a 7 days treatment with cocaine 5 mg/kg, i.p., but are in accordance to [8]. These authors measured changes in the density of muscarinic receptors in rats, 21 days following two exposures to cocaine which was administered continuously for 5 days via sub-cutaneous pellets. Their results showed a reduction of [^{3}H]-quinuclidinyl benzilate binding in various brain areas, including the anterior cingulate cortex and ventral thalamus.

Conclusion

Cocaine administration induces alterations in brain chemistry that lasts after drug withdrawal.

Acknowledgments

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BI-15 EFFECT OF VITAMIN E ON HEPATIC ENZYMES IN MICE INFECTED WITH *Schistosoma mansoni*

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Introduction

Schistosomiasis, a tropical disease caused by the helminth *Schistosoma mansoni,* remains one of the most prevalent infection in many countries, with about 200 million people infected throughout the world [1]. The morbidity and mortality as well as the transmission continues at high levels due to the lack of vaccines, the failure to eradicate the mollusc vector and the recent development of parasite resistance to antischistosome drugs [2]. This injurious disease is characterized by granulomatous reactions around the eggs trapped in the host tissues which leads to hepatic fibrosis, portal hypertension, gastrointestinal hemorrhage and, in some cases, death [3].

In the liver, inflammatory cells, mainly eosinophils (50%) and to a lesser extent, macrophages (30%) and lymphocytes, gather at the site of egg loading and release cytotoxic proteins. The large number of eosinophils present in the granuloma can produce reactive oxygen intermediates, such as O_2^-, H_2O_2, HO^- and related species [4, 5]. These oxidant species cause tissue damage, decrease the reduced glutathione and the activity of the H_2O_2–scavenging enzymes: catalase and gluthatione peroxidase [5]. There are evidences that vitamin E interferes with the production of free radicals in some cells [6]. In the present work we used vitamin E as an antioxidant agent to investigate whether it induces a protective effect on the liver from mice infected by *S. mansoni*.

Material and Methods

- **Chemicals**

GSH, glutathione reductase, NADPH, cumene hydroperoxide, bovine serum albumin, folin reagent and hydrogen peroxide were obtained from Sigma Chemical Company (St Louis, USA). Others reagents were purchased from Merck S.A.

- **Treatment and samples collection**

Two groups of male Swiss white mice (*Mus musculus*) eight week-old were infected with 70 cercaria from the São Lourenço da Mata (SLM) strain of *S. mansoni*. One of the groups received orally 0.2 UI of vitamin E, 3 days per week. The animals were killed 55-65 days after infection and the livers removed and homogenized in ice-cold KCl 1.15%. An aliquot of the liver homogenate was removed and the left material centrifuged at 1.500×g, 20min at 4ºC. The supernatant was centrifuged at 24.000×g, 30 min at 4ºC to isolate the mitochondria rich-fraction, and further centrifuged at 68.000×g, 60 min at 4ºC to separate the microsomes and the citosolic fractions [7].

- **Protein and enzyme assay**

Catalase and glutathione peroxidase activities in the samples were assayed by the method of Aebi and Paglia & Valentaine [8,9], respectively. The protein concentration were determined by the method of Lowry *et al.* [10].

- **Statistical analysis**

The results are presented as the statistical mean ± SD and data for groups were compared by the Student *t*-test for unpaired samples.

Results and Discussion

Significant differences ($p<0.05$) were observed in both enzymes, catalase and glutathione peroxidase in the group supplemented with vitamin E in comparison with the group without vitamin E supplementation. Catalase function in cells eliminating the hydrogen peroxide and producing oxygen and water [11]. Catalase activity in the mitochondria and microsomes rich-fractions of the liver was significantly reduced by about 35% ($p<0.05$) in the vitamin E supplemented group, compared to the controls (Figure 1). Glutathione peroxidase is a remarkable enzyme, which removes from the cells toxic peroxides formed in the normal course of growth and metabolism under aerobic conditions, protecting the cells from the deleterious effects of reactive oxygen intermediates produced during infectious disease [12]. Glutathione peroxidase activity on the supernatant and mitochondria rich-fraction of the liver from the supplemented animals showed a significant increase ($p<0.05$), by 10 to 15%. However, on the citosolic and microsomes rich-fractions from the same animals the enzyme activity was reduced ($p<0.05$) by 37% and 12%, respectively. (Figure 2). Total protein were significantly higher ($p<0.05$) in cellular fractions of the liver in those animals which were supplemented with vitamin E (data not presented) suggesting that the treatment can ameliorate the protein solubility in liver, maybe, due to a reduction on the liver fibrosis, where a great number of proteins are immobilized. The ratio liver/body weight was significantly lower ($p<0.05$) in vitamin E supplemented mice, which showed a reduction by 10.6%.

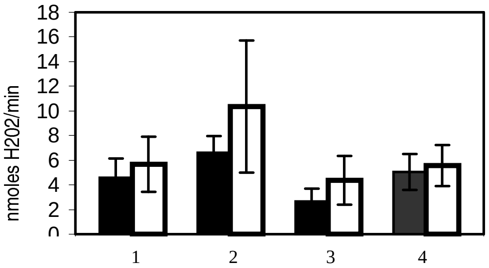

Figure 1 – Catalase activity in Schistosomiasis. Liver fractions of mice infected with *Schistosoma mansoni* and supplement with vitamin E (solid boxes) and without vitamin E supplementation (open boxes). Cell fractions: 1-supernatant, 2-mitochondria rich-fraction, 3-microsomes rich-fraction and 4-citosolic fraction.

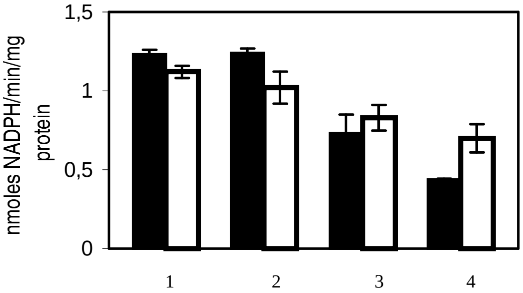

Figure 2 – Glutathione peroxidase activity in Schistosomiasis. Liver fractions of mice infected with *Schistosoma mansoni* and supplement with vitamin E (solid boxes) and without vitamin E supplementation (open boxes). Cell fractions: 1-supernatant, 2-mitochondria rich-fraction, 3-microsomes rich-fraction and 4-citosolic fraction.

Conclusion

This results suggest that vitamin E supplementation modify the activity of liver enzymes involved in the antioxidant mechanisms. It was observed a lower activity of catalase in all the cellular fractions obtained from the supplemented animals, which may reflect a vitamin E protective effect in schistosomiasis. However, the increased glutathione peroxidase activity in the supernatant and mitochondria rich-fraction suggest a different distribution of the enzyme on cellular compartments in response to the treatment.

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BI-16 EVIDENCE FOR AN ANTI-INFLAMMATORY RESPONSE TO TREATMENT WITH A NEW PHTHALIMIDE DERIVATIVE *N*-(2-NITROPHENYL)-PHTHALIMIDE

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Introduction

Phthalimide derivatives are an interesting class of compounds due to their biological activities, for example: anti-convulsant [1], antiviral [2], anti-inflammatory [3]. They also have been shown to be potent hypolipidemic agents in rodents [4, 5].

Phthalimide derivatives may be obtained by the reaction of phthalic anhydride with amines. Among the several methods used for screening and evaluation of anti-inflammatory drugs, one of the most commonly employed techniques is based upon the ability of such agents to inhibit the edema produced in the hind paw of the rodent by injecting an inflammatory agent [6]. The doses of anti-inflammatory drugs, which have been used to demonstrate inhibition of edema, are mostly within the toxic range [7].

The objective of this work was to evaluated whether *N*-(2-nitrophenyl)phthalimide, a new phthalimide derivative, has *in vivo* anti-inflammatory activity.

Experimental methods

• Animals

Young (three months old) male Swiss white mice, having 25 to 30 g body weight, were maintained with water and food (Labina – Agribands of the Brazil Ltda.) *ad libitum*. In the experiment 50 mice were split up into 5 groups of 10 animals.

• Drugs administration

The drugs used were phthalimide and *N*-(2-nitrophenyl)phthalimide. The compounds were suspended in 1% carboxymethylcellulose and administered intraperitoneally, in the morning [7], at a drug concentration of 20 mg/kg/day, for 16 days. Other group received 1% carboxymethylcellulose.

• Anti-inflammatory activity

A positive and negative anti-inflammatory tests were done in two groups by intraperitoneal administration of 250 mg of aspirin (standard for pharmacological comparative tests [8]) and 0.9% of aqueous saline solution, respectively. The anti-inflammatory activity was determined by Levy's method [9]. 0.1 mL of 1% Carrageenin (Sigma, St. Louis, USA) in 0.9% NaCl was injected through the plantar tissue of the right hind paw of each mouse to produce inflammation. After four hours their paws were cut and weighted. The results were analysed according to percentage of the reduction of inflammation, as described earlier [10].

• Statistics

The results are expressed as mean ± S.D., and the significance of the difference between the control and test groups was evaluated by the Student ‘t’ test for independent samples. In all cases, $p \leq 0.05$ was used as the criterion of statistic significance.

Results and discussion

Phthalimide reduced by 12% the carrageenin-induced edema in Swiss white mice when compared to the control group, but this effect was not significant (Figure 1). However, when the hydrogen atom was substituted by a nitro group in the *ortho* position of the phenyl ring and resulted in the compound *N*-(2-nitrophenyl)phthalimide, the anti-inflammatory activity increased by 52%, and it was significantly ($p \leq 0.05$) different from the groups with saline and carboxymethylcellulose (Figure 1). Furthermore, the anti-inflammatory effect of *N*-(2-nitrophenyl)phthalimide was similar to that obtained with aspirin (Figure 1). Our results were similar to previous reports in which other compounds, such as oxadiazole, acid-3-[3-(phenyl)-1, 2, 4, oxadiazole-5-yl] propionic, demonstrated anti-inflammatory activity, the edema in rodents was reduced by 43% at dose of 75 mg/kg [11].

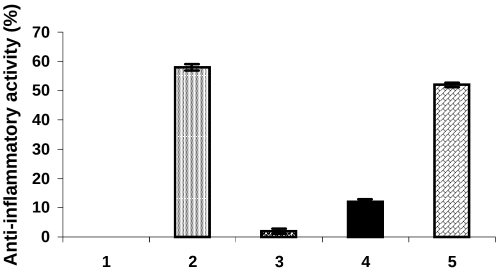

Figure 1. Anti-inflammatory effect of the phthalimide and *N*-(2-nitrophenyl)phthalimide in Swiss white mice after treatment with 1% carrageenin in 0.9% NaCl. **1**, 0.9% NaCl; **2**, aspirin; **3**, 1% carboxymethylcellulose; **4**, phthalimide; **5**, *N*-(2-nitrophenyl)phthalimide.

Conclusion

This study showed that the new phthalimide derivative, *N*-(2-nitrophenyl)phthalimide was able to reduce the carrageenin-induced edema in mice. Therefore, it may offer a good future promise as a new anti-inflammatory agent.

Acknowledgements

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BI-17 INFLUENCE OF MONOOLEIN ON *IN VIVO* PROTOPORPHYRIN IX ACCUMULATION IN HAIRLESS MOUSE SKIN INDUCED BY 5-AMINOLEVULINIC ACID

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Introduction

Photodynamic therapy (PDT) is an experimental modality of cancer treatment, which uses a photosensitizer localized to a tumor tissue where it can produce tissue destruction by absorbing an adequate dose of light of appropriate wavelength to induce cell death via formation of free radicals. A new technique for PDT based on the heme biosynthetic pathway was recently introduced [1]. 5-aminolevulinic acid (5-ALA) is prodrug that can be converted in situ by the heme biosynthetic pathway, into a effective photosensitizer, protoporphyrin IX (PpIX) [2]. A limited 5-ALA penetration into the deep layers of the lesions and lack of seletictive accumulation of 5-ALA-induced PpIX in skin tumors are some reasons for the unsatisfactory results of the treatment. Therefore, methods to improve the efficacy of 5-ALA-PDT are being sought, like supplemented 5-ALA with the chemical enhancer [3].

Monoolein (MO), glycerol monooleate, has a great interest in pharmaceutical formulations as a penetration enhancer and due to its amphiphilic characteristic that facilitate the incorporation of both hydrophilic and hydrophobic molecules. In addition, it is non-toxic, biodegradable, biocompatible and generally recognized as safe, differently of the most penetration enhancers that are frequently used [4].

Objectives

This work is a study of the MO influence on the *in vivo* skin accumulation of PpIX after application of different formulations containing 5-ALA.

Methods

- **Formulations applied**

The formulations used in these studies were 5% (w/w) 5-ALA, 0 or 20% (w/w) MO in propylene glycol. It is used too four controls: untreated skin; treated skin with propylene glycol; treated skin with MO; and treated skin with 20% (w/w) MO in propylene glycol. Experimental formulations were applied on the dorsal region of hairless mice for 2 hours. Animals were sacrificed and treated skin areas were used for PpIX quantitative extraction. All procedures were performed under subdued light.

- **PpIX quantitative extraction**

Briefly, treated hairless mouse skin areas weighing approximately 0.2 g were homogenized in 25 ml of methanol/water mixture (9:1 v/v), sonicated for 15 min. The fluorescence of the supernatants was measured spectrofluorimetrically at λ excitation/emission = 400/632nm [3]. PpIX concentrations were estimated from a standard curve prepared with solutions containing known concentrations of PpIX. PpIX of skin was expressed as µg PpIX/g skin weight.

Results and Discussion

The accumulation of PpIX induced by 5-ALA in hairless mouse skin is represented in Figure 1.

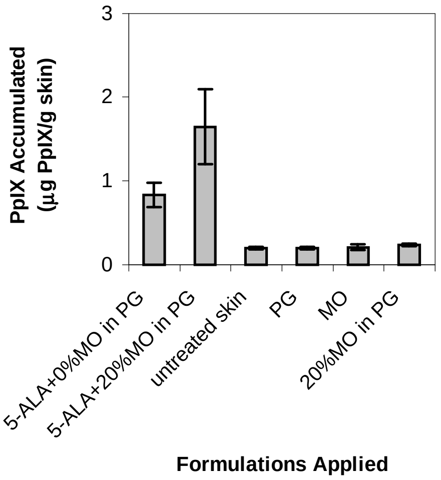

Figure 1: PpIX accumulation in hairless mice skin following topical application of different formulations.

A significant increase ($p<0.05$) of the amount of PpIX extracted from skin after application of 5% 5-ALA (w/w) with 20% MO (w/w) in propylene glycol was observed when compared with the effect of the formulation containing only 5% 5-ALA (w/w) in propylene glycol.

Untreated skin and control formulations without 5-ALA showed similar accumulation of PpIX indicating that these formulations caused no production and accumulation of PpIX into the skin. Our previous work [5] proved the enhancer effect of MO at 20% (w/w). It was found an increase of the *in vitro* flux of 5-ALA through hairless mouse skin in an order of 35 folds compared to a control formulation (Table 1).

Table 1. Skin permeation parameters of 5-ALA in different formulations [5]:

Formulation	**Fluxes J ($\mu g/cm^2$/hours)**
5-ALA5.0% (control)	13.98
5-ALA5.0% +20.0%MO	486.92

However, the increase of *in vivo* PpIX accumulation by MO found in the present work represents the most important data for the optimization of PDT-5-ALA topical.

Conclusions

Appropriate PpIX production and accumulation in tissues could be a crucial factor for the success of PDT. The results obtained in the present work lead to consider that the presence of MO could increase the PpIX accumulation in hairless mouse skin, after topical administration of 5-ALA.

Acknowledgements

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BI-18 PLASMA HDL-CHOLESTEROL ENRICHMENT UPON TREATMENT WITH *N*-PHTHALIMIDOMETHYL α-D-MANNOPYRANOSIDE AND ITS DERIVATIVES

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Introduction

In our research on the synthesis of monosaccharides, we have been interested in the preparation of novel D-mannose derivatives and recently, we wanted to incorporate phthalimide group in mannose. The reason for such a synthesis is that *N*-substituted phthalimides are biologically active. For example, some phthalimide derivatives are antitumor [1], anticonvulsant [2], and hypolipidaemic [3,4]. The literature does not record a phthalimidoylalkyl moiety as an aglycone in monnopyranoside.

Several of the extrahepatic manifestations of liver diseases, are exacerbated by abnormal plasma lipoproteins with altered lipid and apolipoprotein compositions. The abnormal circulating lipoproteins have an adverse effect on the process of reverse cholesterol transport, and following interaction with cell membranes, induce cellular dysfunction either indirectly by altering membrane lipid composition, which modulate cellular ion transport, enzymatic and receptor properties, or directly through apolipoprotein-mediated effects [5]. This paper reports the analysis of the cholesterol content in the high density lipoprotein (HDL-cholesterol) from plasma of mice treated with compounds **a**, **b**, **c**, and **d** (Figure 1).

Experimental methods

Materials

An Electrothermal digital melting point apparatus (model 9100) was used to determine the melting points. Infrared spectra were measured with a Bruker model IFS66 FT-IR spectrophotometer using potassium bromide discs. NMR spectra were recorded with a Varian Unity Plus 300 MHz instrument using $CDCl_3$ as solvent, unless otherwise stated, and tetramethylsilane as an internal standard. Silica gel coated plates with fluorescent indicator (PF_{254}) were used for thin-layer chromatography (TLC) and the spots were revealed under ultraviolet light. The solvent system for running the TLC plates was a mixture of 1:9 ethyl acetate-dichloromethane

Synthesis of phthalimidomethyl α-D-mannopyranoside and its derivatives.

Phthalimide (**a**), phthalimidomethyl 4,6-di-*O*-acetyl-2,3-dideoxy-α-D-*erythro*-hex-2-enopyranoside(**b**), phthalimidomethyl 4,6-di-*O*-benzoyl-α-D-mannopyranoside (**c**) and phthalimidomethyl 2,3-di-O-acetyl-4,6-di-*O*-benzoyl-α-D-mannopyranoside (**d**) (Figure 1) were synthesized following the method described earlier [6], and their structures were deduced by spectroscopic means.

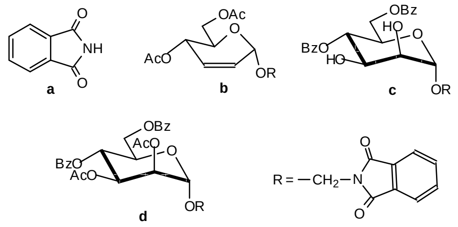

Figure 1: Derivatives of *N*-phthalimidomethyl α-D-mannopyranoside

- ## Lipid Analysis

The compounds were suspended in 1 % carboxymethylcellulose (CMC) and administered orally in the morning [7] at either 10 or 20 mg/kg/day for two-week periods to normolipidaemic male Swiss white mice (body weight 30-32 g). Blood was collected into tubes containing ethylenediaminetetraacetic acid (EDTA 1 mg/mL, disodium salt), on days 0 and 16 and the plasma was separated by centrifugation at 2,500 *x g* for 10 min. Very low density lipoproteins (VLDL) and low density lipoproteins (LDL) in 100 µL of plasma were removed by precipitation with 10 µL of 4% (w/v) natrium phosphotungstate and 2,5 µL of 2M magnesium chloride. The reaction mixture was agitated and centrifuged for 15 min at 3.000 x g and the supernatant, plasma without low density lipoprotein (LDL) and very low density lipoprotein (VLDL), was separated. Plasma cholesterol was determined by the CHOD-PAP method using the enzymes cholesterol esterase, cholesterol oxidase and peroxidase contained in COLESTEROL COD-ANA reagents (enzymatic-trinder), Cat. 60-2/100 (Labtest Diagnostica S.A., Lagoa Santa – MG - Brazil), according to the manufacturer's instructions.

- ## Statistics

All groups had ten animals and each determination was processed before and after the drug treatment. The results are expressed as mean ± S.D. and we used paired Student 't' test. In all cases, $p < 0.05$ was used as the criterion of statistic significance.

Results and discussion

As shown in Figure 2, the plasma HDL-cholesterol for all compounds tested were significantly increased ($p < 0.05$). The groups of mice treated with phthalimide at 20 mg/Kg/day (Figure 2**, a**) increased by 38% the HDL-cholesterol levels. A similar effect was found for phthalimidomethyl 4,6-di-*O*-acetyl-2,3-dideoxy- α-D-erythro-hex-2-enopyranoside (Figure 2**, b**) at 20 mg/Kg/day; plasma HDL-cholesterol was reduced by 39%. However, phthalimidomethyl 2,3-di-*O*-acetyl-4,6-di-*O*-benzoyl-α-D-mannopyranoside (Figure 2, d) at similar dose (20 mg/Kg/day) increased the plasma HDL-cholesterol only by 27%. Surprisingly, the HDL-cholesterol was increased by 23% in animals treated with phthalimidomethyl 4,6-di-*O*-benzoyl- α-D-mannopyranoside (Figure 2**, c**) at half of the dose (10 mg/Kg/day) used with all the other three drugs. Finally, it is important to report that administration of 1% carboxymethylcellulose (Figure 2, CMC) to the animals had no significant changes in mice plasma HDL-cholesterol (3%). There is a growing consensus that elevated levels of HDL-cholesterol have a strong inverse association with the incidence of coronary artery disease [8].

Conclusion

The results show that incorporation of phthalimide group in D-glucal, which resulted in compound **b**, **c** and **d,** was good enough to induce a significantly increased effect in plasma HDL-cholesterol and that might be potential candidate as new hypolipidaemic agents.

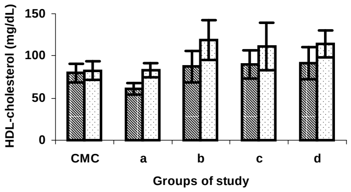

Figure 2 Effect of the compounds CMC, **a**, **b**, **c** and **d** on mice plasma HDL-Cholesterol levels. Before (▨) and after (░) treatment for 16 days.

Acknowledgements

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BI-19 ADJUVANT EFFECT OF CORN AND OLIVE OILS IN MOUSE IMMUNE RESPONSE

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Introduction

Adjuvants are considered as a key issue in the vaccination procedures and adjuvancity is related to the capacity to stimulate the T-cell subsets in charge of the main aspects of the immune response: $CD4^+$ T_H1 and T_H2 and $CD8^+$ cells [1].

A large variety of compounds has been used as immunoadjuvants and among them the mineral oils have a special relevance. The water-in-oil adjuvant vaccines are largely used [2]. However, several inconvenient are related with the mineral oils what stimulates the search for other alternatives. In this way the attempts have been done with vegetable oils [3] that have also to be carefully examined since they may cause contact allergic reactions.

The aim of this work is the evaluation, followed by a careful statistical analysis, of the immunoadjuvant effect of two vegetable oils, olive and corn oil in comparison to the adjuvant effect of a mineral oil (marcol) and of alum ($Al(OH)_3$) in mice immunized with ovalbumin

Experimental methods

- **Materials**

Commercial edible olive (Gallo) and corn (Mazola) oils were used after microbiological analysis and determination of fatty acid content. Marcol.52 (Exxon) and Ovalbumin (Sigma).

- **Immunization**

Groups of 10 swiss mice were immunized by subcutaneous injection with 10 μg OVA (control) and with 10μg OVA associated either to marcol or alum or olive and corn oils. The mice were bled from the orbital plexus on days 7, 14, 21, 28, 35, 42 after the first injection. Booster injections were done on 21st and 35th day in the same conditions as in primary immunization.

- **Antibody assay**

The antibody (IgG1, IgG2a, IgG 2b, IgG3, IgA and IgM) levels raised against OVA were assayed by ELISA (titres) and expressed as log 2 of the inverse of the dilutions starting from 1:10.

Statistical analysis

Data were analyzed by the general linear mixed model (GLMM) which has the equation $Y = X\beta+Zu+e$ [4]. In this equation u is distributed multivariate normal with mean vector 0 and covariance matrix G (which we denote MVN (O,G)), and e is distributed MVN (O,R). The only requirement of the matrices G and R is that they be positive definite (that is, they are covariance matrices). Then, the covariance matrix of Y is $V(Y) = ZGZ' + R$. G is defined as a random factor whereas R is repeated factor, in our case oil is a random factor and time of measurement is our repeated measure. Note that under this mathematical model, Y is distributed MVN ($X\beta$, $ZGZ' + R$) thus the generalized least squares estimates of β is $(X'V)^{-}XV^{-1}Y$. Under this mathematical scenario we have a repeated measure structure. The objectives of repeated measure data analysis usually are to compare treatment means or treatment regression curves over time. A lot of effort, however, is usually needed at the beginning of the statistical analysis to assess the covariance structure of the data. We used the Akaine's Criterion to judge the best fitting covariance structure. The best covariance structure to our data was "no structure" that is, no mathematical pattern was imposed on the covariance matrix.

Results and discussion

Comparisons between the immune response induced by OVA alone (control) and OVA associated either to marcol, $Al(OH)_3$ or corn and olive oil are displayed and statistically analyzed in Table 1 where are shown the adjuvant effect of each of them. Marcol, known to activate T_H1 cells had the highest effect followed by identical effect of both oils and the slightest effect of alum that activates T_H2 cells. The comparisons between control and treatments are all significantly larger than the control.

The kinetics of anti-OVA IgG antibody response is shown in Figure 1. In primary reaction the adjuvant effect is observer after the 14th day and marcol exerted already a stronger effect than the other adjuvants. After the first booster, on 35th day, only with marcol as adjuvant, the ELISA titers diminished in a more pronounced way. The profile of the antibody production in the presence of mineral and vegetable oils show a tendency to increase along the time while in the presence of alum the antibody titers apparently are kept in a plateau. These data suggest a different mechanism of adjuvant effect for the oils, either mineral or vegetable.

Preliminary experiments (not shown) have revealed that olive oil subcutaneously injected is able to promote the migration of eosinophyls among the cells of the influx established after the oil injection.

In spite of the adjuvant effect exerted by the vegetable oils further experiments are needed before they could be safely indicated as immunoadjuvant in vaccination procedures.

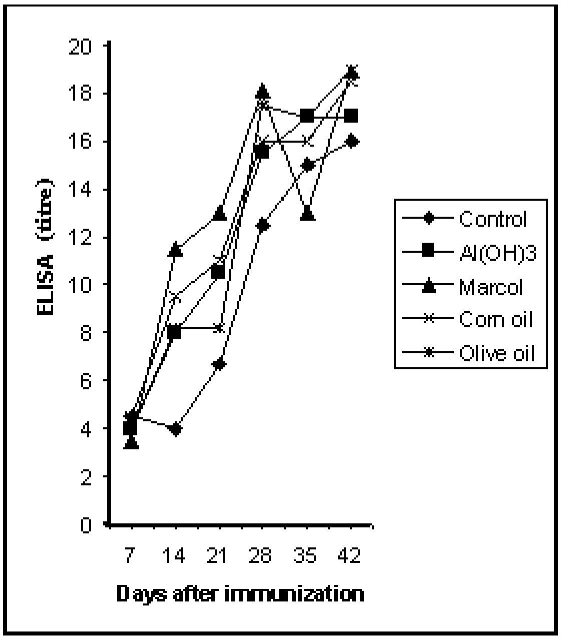

Figure 1: Kinetics of anti-OVA IgG production.

Operating conditions: synthesis of specific IgGs was detected by ELISA in mouse immunized with: ovalbumin (control); ovalbumin plus marcol; ovalbumin plus $Al(OH)_3$, ovalbumin plus olive oil and ovalbumin plus corn oil. Booster injections were carried out 21 and 35 days after primary immunization. ELISA titers are expressed as log 2 of the inversion dilution starting from 1:10.

Conclusion

Vegetable oils of corn and olive exerted an immunoadjuvant effect when injected associated with ovalbumin as antigen. This effect is lower than that observed in same conditions with a mineral oil but higher than the effect exerted by alum.

Table 1
Statistical analysis of immunoadjuvant effects of vegetable oils

ESTIMATES					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Control vs marcol	-3.2500	0.2691	115	-12.08	< .0001
Control vs $Al(OH)_3$	-1.6125	0.2691	115	-5.99	< .0001
Control vs corn oil	-2.2375	0.2691	115	-8.31	< .0001
Control vs. olive oil	-2.2375	0.2691	115	-8.31	< .0001

DF: degrees of freedom
Pr: probability

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BI-20 EVALUATION OF ANTI-INFLAMATORY ACTIVITY FROM *Cratylia mollis* LECTIN IN MICE

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Introduction

Lectins are polyvalent proteins of non-immune origin, which agglutinate polysaccharides or glycoconjugates in a non-covalent fashion. They are ubiquitously distributed in Nature, although found primarily in seeds of leguminous plants [1]. By virtue of their specific carbohydrate-binding properties, lectins have been widely used as laboratory tools in Biology.

Camaratu bean (*Cratylia mollis*) is natural forage from the Semi Arid Region of the State of Pernambuco, Brazil, and belongs to the Fabaceae family. Lectin isoforms (Iso) from the seeds of *C. mollis* (Cra) were obtained: Cra Sephadex and Cra Iso 1. Concanavalin A, Con A, was isolated from *C. ensiformis*, also a Fabaceae. The latter lectin was used as an international pattern and is commercially available. Both lectins are specific to glucose/mannose.

The aim of this work was to evaluate the anti-inflammatory activity of *C. mollis* lectin preparations (Cra Sephadex and Cra Iso 1) in male and female mice.

Experimental methods

• Lectin preparations

Cra lectins were purified according to the protocol reported in [2]. A 10 % (w/v) extract of *C. mollis* seeds (washed with distilled water and powdered) in 0.15 M NaCl (4 °C, 16 h) was ammonium sulphate fractionated, and dialysed against 0.15 M NaCl (F 40-60 %). Following, affinity chromatography was performed in Sephadex G-75, eluted with 0.3 M glucose in 0.15 M NaCl (Cra Sephadex, containing Cra Iso 1 and Cra Iso 4) and ion exchange chromatography in CM-Cellulose, eluted with 0 to 0.4 M NaCl gradient in citrate phosphate buffer (Cra Iso 1). Con A was obtained from Sigma Chemical Company Ltd., St. Louis, U.S.A.

• Anti-inflammatory activity

To adult male mice (30 g), *Mus musculus*, were administered 100 µl of 0.1 % carregeenan solution, in the posterior paw. After 30 min, each animal was treated with acetylsalicylic acid, AAS (250 mg/kg), Cra Sephadex (200, 500 and 1000 µg/animal), Cra Iso 1 and Con A (200 µg/animal). The female mice were treated only with Cra Sephadex (200 and 500 µg/animal). The animals were sacrificed after 4 h from the injection of these products and paws were weighted.

Results and discussion

The results showed a plantar reduction (83.6 % in relation to the control, 100 %) when 500 µg/animal of Cra Sephadex were used. To AAS the reduction was 54.4 % (Figure 1). In male animals Cra Iso 1 and Con A (200 µg/animal) resulted in 76.2 and 75.1 % of the plantar reduction, respectively; the results were similar to Cra Sephadex to the same dose of protein.

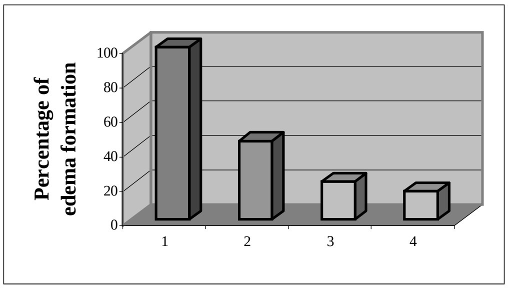

Figure 1 - Anti-inflammatory activity from *C. mollis* lectin preparations in male mice
1- Control; 2- AAS (7.5 mg/animal); 3 - Cra Sephadex (200 µg/animal); 4 - Cra Sephadex (500 µg/animal).

Cra Sephadex was dose dependent and the plantar reduction was more effective at the male mice (83.6 %) in relation to 46.4 % (500 µg/animal) and 73.9 % (1000 µg/animal) with the females (Figure 2).

The assays with male mice showed that all lectin preparations were more effective than AAS (treatment pattern); however, to female mice only 200 µg/animal was higher.

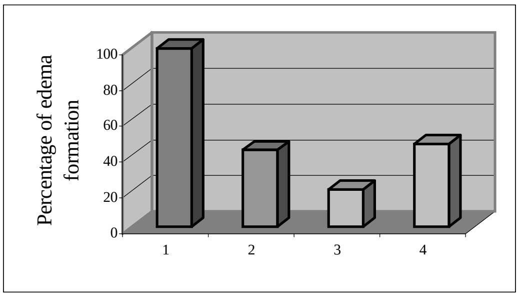

Figure 2 - Anti-inflammatory activity from *C. mollis* lectin preparations in female mice
1- Control; 2- AAS (7.5 mg/animal); 3 - Cra Sephadex (200 µg/animal); 4 - Cra Sephadex (500 µg/animal)

Soybean (*Glycine max*) agglutinin, specific to N-acetyl-galactosamine revealed inflammatory and anti-inflammatory effects after systemic administration [3]. Studies with six glucose/mannose lectins, which also belonged to the Fabaceae family, showed that the carrageenan (300 µg)-induced paw edema was reduced by *Escherichia coli* endotoxin and lectin treatments [4]. An assay with three lectins of different carbohydrate binding affinities revealed that the N-acetyl-glucosamine and glucose/mannose specific lectins had anti-inflammatory activities but the galactose specific lectin was not anti-inflammatory [5]. Cra, specific to glucose/mannose, could induce a blockage of the hypersensibility system, acting at mastocyte cell level and could have an action through the modulation of estrogen hormones.

Conclusion

This study revealed that *C. mollis* lectin was able to reduce paw edema, showing anti-inflammatory activity.

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BI-21 MALONDIALDEHYDE AND CONJUGATED DIENES LEVELS FROM *Schistosoma mansoni* INFECTED PATIENTS AFTER SURGICAL AND CLINICAL TREATMENT

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Introduction

The lipid peroxidation is a complex process where polyunsaturated fatty acids (PUFA) and other lipids are oxidized by intermediates free radicals [1]. This process form conjugated dienes, malondialdehyde and lipid hydroperoxides, among others products [2], which probably are involved in cellular damage [3]. Malondialdehyde is the most abundant aldehyde produced during lipid peroxidation [4] and its determination by thiobarbituric acid is the most generally used test in the lipid peroxidation studies and stress oxidative in diseases [5]. The conjugated dienes results from the formation of lipids radicals; the rearrangement of the double bonds produces the conjugated dienes [1].

Helminthes infect more than one quarter of the word's population and schistosomiasis mansoni is a major health problem, mainly occurring in developing countries [6]. Moreover, young patients showed high prevalence of the hepatosplenic form of the disease [7]. *Schistosoma mansoni* causes liver disease by inducing granulomatous inflammation in this organ, following the loading of eggs. Inflammatory cells release active peroxidase around parasite eggs and *in vitro* studies suggested that the host's response might involve the production of oxygen free radicals [3,8] involved in lipid peroxidation [1]. In the most serious form of the disease the main elective surgical treatment for schistosomiasis patients is splenectomy associated with obliterative suture of the esophageal varices and ligature of the left gastric vein [9]. The aim of this study was to evaluate the degree of lipid peroxidation by measuring the conjugated dienes and malondialdehyde levels in hepatosplenic *Schistosomiasis mansoni* patients who had undergone the surgical and clinical (oxaminiquine) treatments.

Material and Methods

- **Chemicals**

Thiobarbituric acid, malondialdehyde and methanol were obtained from Sigma (St. Louis, USA). Dichloromethane and trichloacetic acid were obtained from Vetec (Rio de Janeiro, Brazil).

- **Malondialdehyde and conjugated dienes assays**

Malondialdehyde and conjugated dienes concentration in all the collected samples were determined as described by Buege and Aust [1].

- **Biological samples.**

The *Schistosoma mansoni* infected group consisted of 18 patients from the "Hospital das Clínicas-UFPE" which were subjected to treatment with oxaminiquine and underwent splenectomy, ligature of the left gastric vein and auto-implantation of spleen tissue. Control group consisted of 18 high school healthy students (same age as the patients) from Recife/PE – Brazil. The study was approved by the Committee on Ethics in Research from the Clinical Hospital at UFPE (Protocol 193/99-CEP/CCS).

Results and Discussion

Malondialdehyde concentration in plasma from patients treated clinically with oxaminiquine (an antischistosome drug) and surgically was increased by 10%, but this rise was not significant (Figure 1). Similarly, Shaheen *et al.* [10], who clinically treated a group of Schistosoma-infected mice with praziquantel (a largely used drug in *Schistosomiasis mansoni* treatment), did not found significant difference in plasma malondialdehyde levels from the infected animals group. According to Esterbauer and Cheeseman [4], malondialdehyde is the most important indicator of lipid peroxidation. Considering that the host response to parasite eggs induces the formation of free radicals [3,8], the low lipid peroxidation, measured as malondialdehyde levels found in this work may reflect a good response to the surgical and clinical treatment. Previous work suggests that the surgical treatment ameliorate the patients' general conditions, such as an increase in the body weight and an improvement of the bone mineral density [9]. On the other hand, significant difference ($p<0.05$) was found in erythrocytes conjugated dienes level between both groups. The patients group showed high level of conjugated dienes (Figure 2).

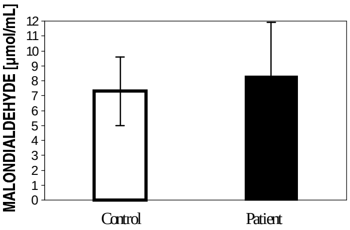

Figure 1 - Plasma malondialdehyde level in *Schistosomiasis mansoni.* Control group (open box) and patients subjected to surgery and ligature of the left gastric vein (closed boxes).

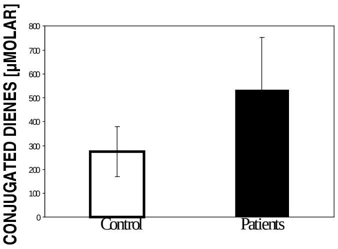

Figure 2 - Plasma conjugated diens level in *Schistosomiasis mansoni.* Control group (open box) and patients subjected to surgery and ligature of the left gastric vein (closed boxes).

Schistosomiasis mansoni can induce the formation of certain types of reactive oxygen species [8], such as superoxide ions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) [11], which induce the lipid peroxidation measured as conjugated dienes level. It is known that conjugated dienes formed by free radicals are able to produce malondialdehyde or lipid hydroperoxide [1]. As observed by the high level of conjugated dienes found in this study, the lipid peroxidation in patients might be higher than in controls, although this was undetected by analysis of the malondialdehyde level.

Conclusion

The surgical and clinical treatment of *Schistosomiasis mansoni* may interfere in the pathway of conjugated dienes, which should be involved in rather the production of another species indicators of cell oxidative stress, such as lipid hydroperoxide, than malondialdehyde.

Acknowledgements

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BI-22 INHIBITION OF HUMAN PLATELET AGGREGATION BY HYDROALCOHOLIC EXTRACT AND COUMARIN FROM *Torresea cearensis* Fr. All.

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Introduction

Torresea cearensis Fr. All., popularly known as “cumaru”, is a plant belonging to the Fabaceae family and commonly found in Northeast of Brazil. It has been demonstrated that coumarin (2H-l-benzopyran-2-one) is one of the main constituent from stem bark of *T. cearensis* Fr. All [1]. The objective of the present work was to study the effect of hydroalcoholic extract (HAE) and coumarin (COUM) from *T. cearensis* Fr. All. on human platelet aggregation and ATP release.

Experimental methods

- **Drugs**

Adenosine diphosphate (ADP), adenosine triphosphate (ATP), epinephrine (EP), yohimbine (YOH) and luciferase-luciferin (LL) were purchased from Sigma, USA. Other drugs were of analytical grade.

- **Plant material**

The plant was collected at the city of Quixeramobim, Ceará, in the Northeastern Region of Brazil. Voucher specimens (no 837 and 847) are deposited at the Prisco Bezerra Herbarium of the Federal University of Ceará. Ground dried stem bark was extracted with ethanol and evaporated (HAE) and the volume brought to the desired concentration before use. Coumarin was isolated by extraction of stem bark with ethanol in a Soxhlet apparatus. The ethanolic fraction was concentrated and mixed with distilled water. This fraction was extracted with hexane, followed by chloroform. Then, the chloroformic extract was chromatographed over silica gel, producing three main fractions that were identified as coumarin, isokaempferide, and flavonoids through spectrometry analysis.

- **Platelet aggregation and ATP release in human platelet-rich plasma**

Platelet-rich plasma (PRP) was obtained from blood collected from the antecubital vein of health human volunteers (with previous consent) who did not take any medication for at least two weeks prior blood collection. The blood was anticoagulated with sodium citrate solution (0.129 M, pH 7.5) (9:1, v/v) and centrifuged for 10 min at 1000 rpm at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation of blood at 3000 rpm, for 20 min. Platelets were counted and adjusted to 260,000/mm^3 using PPP to dilute PRP. Aggregation was performed according to the method of Born and Cross [2] with a Lumi-aggregometer Model 450 from Chrono-Log Co. U.S.A.

ATP release was measured simultaneously by addition of LL (50 μl) to PRP. This reagent becomes luminescent in ATP presence. The luminescence was calibrated by ATP addition.

- **Statistical analysis**

All values were expressed as means ± S.E.M. of maximal light transmission (MLT). Data were subjected to Analysis of Variance (ANOVA) and values of $p < 0.05$ were considered significant.

Results and discussion

ADP is a physiological mediator of platelet aggregation and it is released from dense granular bodies of platelets by various activators [3]. HAE (22 and 44 μg/ml) and COUM (12 and 24 μM) reduced in a dose-dependent manner the ADP (10 μM) induced aggregation, with inhibition rates ranging from 14 to 49 % of MLT (table 1). In the same way, COUM (24 μM) also reduced ATP release (25.2 ± 3.8 mm) when compared to control (109.3 ± 15.0 mm) corresponding to inhibition of 77 %. In order to verify whether COUM behaved as an inhibitor of TXA_2 formation, the effect of COUM was studied after its association with aspirin (ASP). The results showed that ASP inhibition of platelet aggregation was potentiated in presence of COUM (12 μM) (table 1).

Table 1. Effect of HAE, COUM and ASP on ADP induced platelet aggregation.

Group		MLT (mm)	% of inhibition
Control		122.5 ± 5.4	-
HAE	22 μg/ml	101.0 ± 3.7[a]	17.5
	44 μg/ml	72.1 ± 11.3[a]	41.1
COUM	12 μM	105.6 ± 5.7	13.7
	24 μM	63.0 ± 5.2[a]	48.5
ASP	50 μM	97.0 ± 5.2[a]	20.8
ASP +	50 μM		
COUM	12 μM	68.0 ± 4.2[b]	44.4

PRP was incubated with saline (control), HAE, COUM or ASP for 5 min at 37° C. For experiments performed with ASP in the presence of COUM, the latter was added after the first 5 min incubation in the presence of ASP. Incubation then continued for further 5 min until ADP (10 μM) addition. Data are means ± S.E.M. of 4 experiments. a and b ($p < 0.05$) vs. Control and COUM (12μM) respectively (ANOVA).

The effects of HAE and COUM on EP (10 μM) induced aggregation are presented on table 2. HAE (22 and 44 μg/ml) showed inhibition rates of 35 and 50 % respectively of MLT when compared to control group. Similarly, COUM (3 and 6 μM) significantly reduced MLT (97.3 ± 8.9 and 77.0 ± 9.5 mm respectively) when compared to control (118.6 ± 3.8 mm).

Table 2. Effect of HAE, COUM and YOH on EP induced platelet aggregation.

Group		MLT (mm)	% of inhibition
Control		118.6 ± 3.8	-
HAE	22 μg/ml	77.5 ± 6.6[a]	34.6
	44 μg/ml	59.0 ± 7.5[a]	50.2
COUM	3 μM	97.3 ± 8.9[a]	17.9
	6 μM	77.0 ± 9.5[a]	35.0
YOH	1 μM	90.8 ± 8.7[a]	23.4
YOH +	1 μM		
COUM	3 μM	21.3 ± 5.3[b]	82.0

PRP was incubated with saline (control), HAE, COUM or YOH for 5 min at 37° C. For experiments performed with YOH in the presence of COUM, the latter was added after the first 5 min incubation in the presence of YOH. Incubation then continued for further 5 min until EP (10 μM) addition. Data are means ± S.E.M. of 4 experiments. a and b ($p < 0.05$) vs. Control and COUM (3μM) respectively (ANOVA).

Platelets bear α_2-adrenoceptors on its surface and stimulation of these receptors by EP induces platelet aggregation [4]. The possible involvement of α_2-adrenoceptors in the COUM antiplatelet effect was determined by its addition to yohimbine (YOH), a known α_2-antagonist. The effect of YOH was greatly potentiated by COUM (3 μM) (Table 2).

In human PRP, ADP and EP cause biphasic aggregation with secretion accompanying the second phase. Secondary aggregation and ATP secretion are dependent upon the release of arachidonic acid (AA) from membrane phospholipids and formation of proaggregatory cyclic endoperoxides and TXA_2 and is blocked by cyclooxygenase (COX) inhibitors such as ASP. By contrast, the primary wave of aggregation depends upon the interaction of EP with membrane α_2-receptors and is a prerequisite step for the activation of phospholipase A_2 (PLA_2) leading to the release of AA and generation of the secondary wave of aggregation response to EP [5].

Conclusion

The antiplatelet effects of HAE and COUM seem to be mediated by the COX, TXA_2 formation and PLA_2 inhibition.

Acknowledgements

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BI-23 POTENTIAL ANTICHAGASIC PRODRUG OF NITROFURAZONE WITH DECREASED MUTAGENIC ACTIVITY RELATED TO NITROFURAZONE

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Introduction

Chaga's disease represents a serious health problem in at least 17 countries of the American Continent. About 100 million people live at risk of contracting the disease and 16-18 million are indeed infected. The medical and social impact of Chaga's disease is high. In Brazil at least 10% of infected people develop severe cardiac or digestive chronic disease, the medical costs for their obligatory treatment could reach US$ 250.000 million per year. Similarly, the minimum absenteeism of 75.000 Brazilian chagasic workers with serious cardiac damage could represent losses of US$ 5.625,00 per year [5].
Nitro compounds inhibit trypanothione reductase, the key enzyme of the antioxidant metabolism of *T. cruzi*. In addition, these drugs are potential mutagenic agents due to the nitro group in molecule[3]. Only two drugs are available: nifurtimox and benznidazol. Both drugs are used in the acute phase of the disease. In Brazil, only benznidazol is available in the market.
Chung *et al.* (1996) synthesized several nitrofurazone prodrugs [1] and studied their antichagasic effect. They observed an increase in the antichagasic activity of the prodrugs in comparison to nitrofurazone (NF) and to benznidazol.

The objective of the present study was to synthesize a prodrug of nitrofurazone, and investigate its mutagenic effect.

Experimental methods

- **Materials**

Nitrofurazone, hydroxymethylnitrofurazone, potassium carbonate, formaldehyde, acetone, sodium chloride, potassium chloride, magnesium chloride, oxoid midle, biotin, histidine, glucose, NAPD, S9 mix, *Salmonella typhimurium* strains TA102 and TA98.

- **Synthesis of Hydroxymethylnitrofurazone [2]**

Hydroxymethylnitrofurazone (NFOH) was obtained by reacting 5.0 nmol of nitrofurazone (NF) with 18mL of formaldehyde and 5.0 nmol of potassium carbonate plus 10 mL of water. The reaction was shaked for 24 hours. The suspension was filtered, washed with methanol and dried.
Purification was carried out by adding 5.0g of NF in 50 mL of acetone, this mixture was heated and evaporated until recristalization of NF. The precipitate was filtered and dried. The product was identified by melting point, NMR ^{1}H and ^{13}C.

- **Ames Test [4]**

The plates were added with an agar minimum glucose (AMG) and the bacteria were direct inoculated in nutrient broth and maintained at 37°C for 14 hours.
Biotin/histidine solution was added to the top agar (surface agar) and it was homogenized, being distributed in test tubes. To each tube 0.1mL of the inoculum and NFOH (5–10–25–50–100 nmol) were dissolved in DMSO. After homogenization, the tube contents were poured at the surface of a plate with minimum agar. The plates were incubated at 37ºC for 48 hours. The influence of the metabolic activation was tested by adding 500 µL of S9 fraction of mouse liver treated with aroclor 1254. Positive controls: 4–nitro–phenylenediamine (10 g/plate) for the TA98 strain and daunomycin (3.0 g/plate) for TA102 strain – and negative control: 75,0 µL of DMSO – were included. After 48 hours the revertant number was counted and the mutagenic rate (MR – the rate between the average revertant number for each concentration and the average of the number of revertants of the negative control) was calculated.

Results and discussion

NMR ^1H spectra [4.62–4.59(d,CH_2)], IR (ν_{OH}3274.70 cm^{-1}) and melting point 167°C, revealed that the product obtained was the hydroxymethylnitrofurazone.

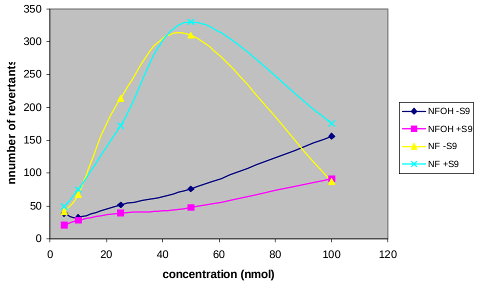

Figure 1: Mutagenic activity of NFOH and NF on TA98 strain with and without metabolic activation

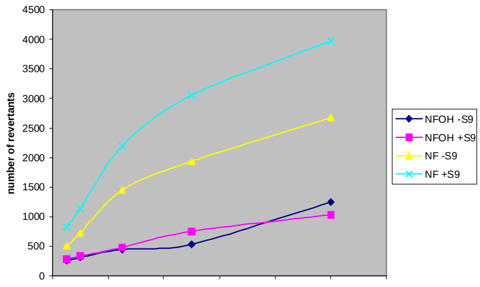

Figure 2: Mutagenic activity of NFOH and NF on TA102 strain with and without metabolic activation

Table 1: Rate of mutagenicity (RM) of NFOH (2) in *Salmonella typhimurium* (TA98 e TA102) with (+) and without (-) metabolic activation (S9).

conc.	Mutagenic Reason per plate							
nmol	TA98				TA102			
	- S9		+ S9		- S9		+ S9	
	NFOH	NF	NFOH	NF	NFOH	NF	NFOH	NF
5,00	1,77	1,66	0,84	1,64	1,24	**2,28**	1,32	**3,60**
10,0	1,47	**2,69**	1,14	**2,50**	1,53	**3,25**	1,59	**4,95**
25,0	**2,36**	**8,56**	1,57	**5,73**	**2,16**	**6,54**	**2,27**	**9,53**
50,0	**3,45**	**12,4**	1,90	**11,0**	**2,61**	**8,72**	**3,52**	**13,27**
100,0	**7,09**	**Toxicity**	**3,66**	**Toxicity**	**6,04**	**12,0**	**4,82**	**17,28**

Obs: The labeled values show mutagenic activity (MR ≥ 2.00).

The decrease in the number of revertants for NF at the maximum concentration was due to the toxic effect of the drug itself (Figure 1 and Table 1). The parent drug (NF) showed mutagenic activity on the TA102 strain (assay –S9) at the smallest concentration (5.0 nmol), while in the prodrug (NFOH) the toxicity was observed at the concentration of 25 nmol.

Considering the assay with S9 fraction of TA98 strain the result showed that mutagenic activity of NFOH was evident at maximum concentration tested (100nmols), while mutagenic activity of NF appeared at the lower concentration (10 nmols). For TA102 strain (+S9 assay) the mutagenic activity of NFOH appeared with 25 nmols while for the parent drug this effect was observed with the smallest concentration tested. Moreover the mutagenic activity of NF was always higher than NFOH in both assays (with and without metabolic activation).
The Ames test showed that NFOH was about 3-4 fold less mutagenic than NF at the same concentrations in assay accomplished with the both strains (Figure 1 and 2).

Conclusion

The results show that the prodrug NFOH was obtained in a high yield (90%) and that the molecular modification performed on nitrofurazone decreased the mutagenic action and increased antichagasic activity.

Acknowledgements

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BI-24 SUSCEPTIBILITY *IN VITRO* OF *SCHISTOSOMA MANSONI* TO IMIDAZOLIDINONE DERIVATIVES

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Introduction

Schistosomiasis is considered the most important of the human helminthiasis in terms of morbidity and infection. It was reported by WHO that (World Health Organization) 200 million people are infected, a further 600 million people are at risk situations in endemic regions and an estimate of 200.000 deaths occurs annually. Anthelmintics are used for the treatment and control of the human schistosomiasis, considering that no vaccines are yet commercially. Resistance to medical anthelmintics has occurred in schistosomes and can be expected to increase concomitantly with continued use of chemotherapeutic agents [1].

The drug discovery and development process takes a long time and is expansive. To design a drug totally from scratch is theoretically possible, but it complex and usually unnecessary. The lead compounds can often be modified and provide starting points for the synthesis of analogues. Niridazole, chemically 1-(5-nitro-2-thiazolyl)-2-imidazolidinone, was employed in the second decade of XX century for the treatment of schistosomiasis infections. Its efficacy is good but it presents serious drawbacks, such us the need for multiple oral doses and the occurrence of side effects. For this reason, it is recommended to synthesize chemical analogues with potential antischistosomal activity [2].

Since chemotherapy is used to control schistosomiasis and because drug resistance has appeared in field isolates and in laboratory strains of schistosomes, this work emphasizes research on imidazolidinone derivatives to potential antischistosomal compounds.

Experimental methods

- **Materials**

The synthesis and physicochemical properties of 3-(4-chloro-benzyl)-5-(4-fluoro-benzylidene)-imidazolidine-2,4-dione (FZ-7) has been described [3]. This compound was synthesized by aldolisation-crotonisation reaction from 4-fluoro-benzaldehyde and 3-(4-chloro-benzyl)-imidazolidine-2,4-dione in the Laboratory of Design and Synthesis of Drugs, Departamento de Antibióticos (Figure 1).

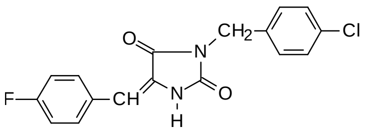

Figure 1 3-(4-chloro-benzyl)-5-(4-fluoro-benzylidene) -imidazolidine-2,4-dione (FZ-7).

- **Determinations of *in vitro* activity of FZ-7**

In vitro models are available, which can be used to study the susceptibility of schistosomes isolates to drugs [4]. The BH (BH - Belo Horizonte, MG, Brazil) strain of *S. mansoni* was routinely maintained in our laboratory by standard passage through *Biomphalaria glabrata.* Adult male and schistosomes, obtained by perfusion of untreated mice infected 8-12 weeks earlier [5] were maintained suspended *in vitro* in RPMI-1640 medium supplemented with HEPES, foetal bovine serum, Penicillin, and Streptomycin. Incubation was carried out at 37°C in 5% CO_2 atmosphere. The effect of FZ-7 on the viability of the *S. mansoni* was examined by incorporating the drug into the medium employed for parasite culture.

The drug at appropriate concentrations (80 and 120 μg/ml) was dissolved in DMSO (1.5% v/v). This solvent concentration did not affect worms mortality, pairing, and motility. In all experiments two adult worm, paired or unpaired were placed in multi-well plastic tissue culture dishes. A minimum of 18 worms was used in each treatment group. Motility, pairing, and mortality was observed under inverted microscope at 15 days after FZ-7 addition. Control worms were treated with DMSO and were included in every experiment. Parasites were considered dead when no movement could be detected over a 3-min period.

Results and discussion

At 80µg/ml, FZ-7 had little effect on parasite mortality and pairing, but produced marked decreases in worm motility within the first 24 hours. The Figure 2 shows that at 120µg/ml was observed reductions in schistosome pairing. FZ-7, as well Niridazole, showed a lethal action more important on female worms than on male *S. mansoni* [6].

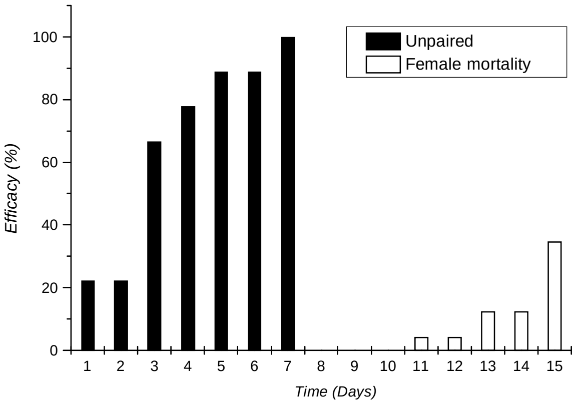

Figure 2 The efficacy of FZ-7 at 120ug/ml against female mortality (white) and unpaired (black) of *S. mansoni* maintained *in vitro* for 15 days

The experiments show that the exposure to the FZ-7 resulted in the death of 35% female and this lethal effect was dependent on drug concentration and time incubation. The degree of cell damage caused by FZ-7 treatment was more severe than the effect observed in worms without drug. It was observed ballooning of the tegument on treated group.

Conclusion

Our results indicate that this compound is deleterious to *S. mansoni* maintained *in vitro*.

Acknowledgements

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BI-25 HAEMAGGLUTINATING ACTIVITY IN TISSUE CULTURES AND ANTIOXIDANT PROPERTIES FROM LEAF PREPARATIONS OF *Bauhinia monandra* (pata-de-vaca, pulse)

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Introduction

The use of plants as therapeutic agents have had a remarkable role in the medicine of different countries.

The genus *Bauhinia* (Fabaceae) includes a number of ornamental species which are well distributed in Brazilian cities. Native or introduced species have been used as forage, human food or as phytotherapics for treatment of diabetes and as a diuretic. Infusions from leaves of *B. monandra* have been frequently used in popular medicine. Haemagglutinating activity (HA) detected in various species of *Bauhinia* has been associated with the presence of lectins. A galactose-specific lectin (BmoLL) was identified in leaves of *B. monandra* [1].

Plant tissue cultures may provide a source to obtain homogeneous substances which could be valuable biotechnological tools. According to investigations carried out in recent years, the prevention of cancer and cardiovascular diseases are associated with the ingestion of fresh fruits, vegetables or teas containing antioxidant compounds. A method has been used to evaluate these compounds, based on the reduction of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) by antioxidant compounds in methanol solution [2]. In its radical form DPPH absorbs at 515 nm, but upon reduction by an antioxidant, this absorption is reduced according to the reaction:

$$\text{DPPH}\bullet + \text{AH} \longrightarrow \text{DPPH-H} + \text{A}\bullet$$

(purple colour) (yellow colour)

In order to study the HA of different types of tissues originated from *B. monandra*, *in vitro* cultures (callus, axenic and *Agrobacterium rhizogenes*-transformed root cultures) were established. Also, the antioxidant activity of five preparations (crude ethanol extract as well as hexane, chloroform, ethyl acetate and methanol:water fractions) obtained from leaves of *B. monandra* was tested.

Experimental methods

- ***B. monandra* tissue cultures**

Sterile plantlets were obtained from seeds of *B. monandra* germinated in Murashige and Skoog medium (MSO) containing sucrose (30 g/L) and agar (10 g/L). Callus cultures were obtained from *B. monandra* leaves (3-month-old sterile plantlets) cultivated in MSO supplemented with 1-naphtaleneacetic acid (NAA; 1 mg/mL) and 6-benzylaminopurine (BAP; 5 mg/mL). Axenic root cultures were established from the plantlets and cultivated in MSO liquid medium supplemented with NAA and kinetin (0.2 mg/mL each). Transformed root cultures were obtained by infection of the plantlets with *A. rhizogenes* LBA 9402, using a hypodermic syringe containing 50 μL of the previously prepared bacteria. The emerged roots were excised and cultivated in MSO liquid medium supplemented with indolbutiric acid (IBA; 2 mg/L) and kinetin (0.1 mg/L), designated MSIC medium and incubated at 100 rpm. All cultures were incubated at 25 ± 1°C, under constant light**.**

- **Extraction of lectins and HA test**

Callus, as well as axenic and transformed roots from *B. monandra* were macerated (10%, w/v) with 0.01 M citrate phosphate buffer containing 0.15 M sodium chloride, pH 6.5, selected buffer, and the homogenate was shaken gently for 16 h at 4 °C. The suspension was submitted to 60% (w/v) ammonium sulphate fractionation (F 0-60%) by addition of solid salt. After 4 h at room temperature, the precipitate was resuspended in the selected buffer and dialyzed against distilled water, followed by the buffer. HA was tested using rabbit erythrocytes and human erythrocytes of blood groups A, B, O and AB treated with glutaraldehyde. The inhibition assay was performed using the sugars D(+)-galactose, D(+)-rafinose, methyl-α-D-mannopyranoside, *N*-acetyl-D-galactosamine, D(-)-fucose, D(-)-fructose, D(+)-xylose, sucrose and D(+)-maltose.

- **Extraction of leaves**

Solvents for the extraction were purchased from Merck. (-)-Catechin and (-)-epi-gallocatechin were adquired from Sigma.

Leaves were finely ground using a multiprocessor and the powder was soaked in ethanol for one week extraction (two times repeated). After filtration the combined extracts were evaporated to dryness to yield the ethanolic extract from *B. monandra* (BmoEtOH). An aliquot (1 g) of BmoEtOH was redissolved in a mixture of methanol:water (3:2 v/v) at room temperature, which was fractionated further with hexane (BmoHex), chloroform (BmoChl) and ethyl acetate (BmoEtAc). The residual methanol-water fraction (BmoMeOH), as well as the other fractions were concentrated under vacuum.

- **Radical Scavenger Capacity (RSC) towards DPPH – Dot blot assay**

BmoEtOH, BmoHex, BmoChl, BmoEtAc and BmoMeOH fractions were redissolved in the respective solvents (0.05 g/mL). A serial dilution (2 to 256) of the stock solution was prepared. The negative controls were pure solvents used for dissolution of extracts; the positive controls were the tannins (-)-catechin and (-)-epi-gallocatechin in methanol (5 mg/mL). A drop of 2 μL of the stock solution and each of the dilutions were carefully placed on a thin layer chromatography plate, TLC (Merck; silica gel $60F_{254}$ aluminium sheets; 20 × 10 cm). The drops were applied in triplicate and left to dry for a few min. The silica sheet was placed up-side down in a 0.4 mM DPPH in methanol for 10 s. Stained silica sheets (purple colour) showed yellow spots where the drops were placed depending on the RSC of the samples. The intensity of the yellow colour depended on the amount and nature of radical scavengers present in the sample [2].

- **Thin layer chromatography**

A drop (2 μL) of BmoEtOH, fractions and catechins were placed individually on the base line of the TLC plate which was developed with eluents A and B [toluene: ethyl acetate: formic acid (8.5:5.0:1.5) and (5.0:4.0:1.0), respectively].

Results and Discussion

- **Evaluation of lectin activity**

HA was detected in the protein fraction (F0-60%) obtained from callus, as well as axenic and transformed root cultures of *B. monandra*, when tested with rabbit erythrocytes and human erythrocytes of blood group AB. The HA of tissues was inhibited with galactose and *N*-acetyl-D-glucosamine. The present work indicates that *B. monandra* tissue cultures provide an interesting model and an alternative supply for the production of homogeneous lectins.

- **Radical scavenger capacity (RSC)**

The dot blot assay (not shown), revealed that BmoEtOH, BmoHex, BmoChl, BmoEtAc and BmoMeOH reacted strongly and quickly with the DPPH solution, almost in the same manner as the potent positive controls (tannins). Qualitative TLC was performed in order to separate the compounds present in the leaf extract (BmoEtOH). The best solvent system for the elution of BmoHex and BmoChl was solvent A. This system revealed the presence of only one compound with antioxidant activity in BmoHex (Rf = 0.88), whilst in BmoChl there were two compounds (Rf = 0.88 and 0.40). BmoEtAc was eluted with solvent B, which revealed the presence of one compound with antioxidant activity (Rf = 0.46). The compounds present in BmoMeOH were submitted to solvents A and B but remained in the base line. The different compounds detected are being separated by column chromatography and their RSC will be tested by a spectrophotometric assay using DPPH. This test will allow the comparison of the kinetic behaviour of the compounds with tannin standards (strong antioxidants). The compounds could be a mixture of fast, moderate and slow antioxidants.

Conclusions

HA was detected in the F 0-60% from the different tissue cultures of *B. monandra*.

The leaves of *B. monandra* contain at least four different antioxidant compounds.

Acknowledgements

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BI-26 STUDY OF THE UPTAKE OF Tc-99M BY RED BLOOD CELLS AND PLASMA PROTEINS OF RATS IN THE PRESENCE OF *PHYLLANTHUS NIRURI*

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Introduction

The use of radionuclides and radio-pharmaceuticals have contributed to the elucidation of cellular systems, their metabolic pathways and enzymatic mechanisms [1,3]. Technetium-99m (^{99m}Tc) is the most used radionuclide, since the 60th decade, due to its optimal physical characteristics [2,3].
Labeling of red blood cells with Tc^{99m} is used in nuclear medicine for determination of various systems, it can be used as an *in vivo* or *in vitro* technique. Studies show the interaction of drugs (natural or synthetic) in blood cells.
Phyllanthus niruri (Euphorbiaceae) is a well-known plant, used in the treatment of renal and hepatic diseases. In Brazil, it received the common name of 'stone-braker', due to its use for elimination of renal calculi and other types of calculi [4].
It is necessary to determinate the radioactivity binding to blood elements, which makes possible to understand how a drug is able to modify the biodistribution of each radiopharmaceutical and to evaluate the specific characteristics of the ligand-binding on the blood cells.
This work aims to label red blood cells and plasma protein with ^{99m}Tc *in vitro.*

Methodology

The labeling experiments were carried out by incubating aliquots (0,5mL) of anticoagulant whole blood, which was withdrawn from Wistar rats. Samples of 250µL were incubated, and gently mixed with 100µL of different dilutions of the *phyllanthus niruri* (0, 2.25, 6.25, 12.5, 25, 50, 100%) and with fractions (G,G1,G2,G3) of the extract of *phyllanthus niruri,* which were separeted by chromatography in filtration gel sephadex G-75, for 60 minutes, at room temperature. The control group received the same treatment. After this period of time, 500µL of a recently prepared stannous chloride solution of 1.2 or 0.6µg/ml were added and the incubation continued for more 60 minutes. Then, ^{99m}Tc-pertechnetate (100µl, 3.7MBq), recently milked from Mo^{99}/Tc^{99} generator (Instituto de Pesquisas de Energia Nuclear, Comissão Nacional de Energia Nuclear), was added and the incubation was continued for another 10 min. These samples were then centrifuged and plasma (P) and blood cells (BC) were separated. Aliquots of 20µL of P and BC were precipitated with 1mL of trichloroacetic acid 5% and insoluble fraction (IF) was isolated. The radioactivity in the samples (P,BC,IF-P,IF-BC) was determined in a gamma counter (DPC Gambyt Cr). The results were calculated as previously described [5,6].

Results and discussion

Figure 1 shows the fixation of the radioactivity in plasma and blood cells from whole blood treated with different concentrations of *Phyllanthus niruri*. The analysis of the results indicates that there is a significant increase ($p<0.05$) in the fixation of ^{99m}Tc in IF-BC in the concentration of 6.2 (from 23.3 to 25.2%) and in the concentration of 25% (from 23.3 to 40.2%) of the extract. In the IF-P, the results indicate that there is a significant decrease in the fixation of ^{99m}Tc in plasma proteins when the concentration of 25% (from 70.7 to 59.2%) and from 50% on, there is an increase.

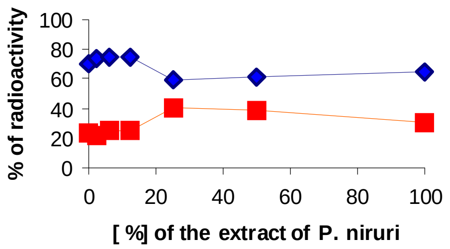

Figure 1. Effects of *P. niruri* extracts in the fixation of ^{99m}Tc on insoluble fraction of plasma and blood cells ($p< 0.05$). n =3(♦ IF-P) (. IF-BC).

The table 1 shows the distribution of the radioactivity in blood cells from Wistar rats blood, treated with chromatographic fractions of *P.niruri*. The results indicate the there is an increase in radioactivity uptake in IF-BC up to the Fraction FG1 (from 24.1 to 60.6%). The results also indicate that in the IF-P there is a significant decrease in the fixation of ^{99m}Tc in plasma proteins when the fraction FG1 (from 75.9 to 39.3) of the referred extract is employed.

Table1 Effect of the chromatographic fractions of *P. niruri* on the$^{\mathbf{99m}}$ Tc uptake by blood cells and plasma proteins.

	% of ^{99m} Tc uptake of chromatographic fractions				
	FC	**FG**	**FG1**	**FG2**	**FG3**
IF-BC	24.1	28.5	60.6	21.9	22.4
IF-P	75.9	71.5	39.3	78.1	77.6

Conclusion

It is concluded that the extract of *Phyllanthus niruri* is able to increase the uptake of ^{99m} Tc in the presence of stannous chloride. It is also concluded that, in low concentration, occurs an inhibition of this uptake, probably, by its oxidant action or by its action at the cellular membrane.

Acknowledgements

To Prof. Mário Bernardo Filho (UERJ) and to the Departments of Pharmacy and Biophysics and Radiobiology that collaborated on the realization of this work.

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BI-27 ANTIBACTERIAL, ANTINOCICEPTIVE AND ANTIEDEMATOGENIC ACTIVITIES OF ETHYL ACETATE FRACTION FROM *Kalanchoe brasiliensis* Camb. (COIRAMA)

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Introduction

Kalanchoe brasiliensis Camb., Crassulaceae, is a medicinal plant commonly known in the Northeast of Brazil as "coirama". Its leaves are used in the treatment of digestive and urinary tract diseases (Magalhães, 1996). Some compounds have been isolated from the leaves of *K. brasiliensis*, as ramnoside patuletin derivates [2]. It was demonstrated that hidroalcoholic extract from this plant had an anti-inflammatory activity, while anti-ulceration and cicatrizated activities were not observed [4]. On the other hand, Coelho *et al.* [1] showed the gastroprotect activity of water extract from the leaves of *K. brasiliensis.* In previous work [3] we detected antinociceptive and antiedematogenic activities in ethyl acetate extract from the leaves of that plant. The objective of the present work was to continue our studies and to evaluate the antimicrobian, antinociceptive and antiedematogenic activities of the ethyl acetate fraction from the leaves of *K. brasiliensis.*

Experimental methods

- **Materials**

Plant material. The authenticated plant material was collected at the Maracanau city, state of Ceará, Brazil. Voucher specimens (No. 17.619) were deposited at the Prisco Bezerra Herbarium, Department of Biology of the Federal University of Ceará.

- **Extraction procedure**

Dried and cut leaves of *Kalanchoe brasiliensis* Camb. (236g) were submitted to extraction with ETOAc at room temperature (22°C) for 24h. The ETOAc extract was concentrated in vacuo to give a residue (30g), which was chromatographed on silica gel using solvents and eluted with n-hexane, $CHCL_3$, ETOAc and MeOH. The EtOAc fraction (EAF; 11.72g) was rechromatographed on silica gel using solvents of increase polarity. The eluates were collected with monitoring TLC to give 29 fraction (EAFFs). The EAFFs 3 (1.51g); 4-8 (3.4 g); 9-11 (406 mg) and 22-29 (19.1 mg) were used in the tests.

- **Pharmacological experiments**

Antibacterial activity. It was determined by diffusion test, using the Mueller-Hinton agar medium and the following microorganisms: *Pseudomonas aeruginosa* (ATCC), *Escherichia coli* (ATCC) and *Staphylococcus aureus* (ATCC). The antimicrobial activity was recorded by inhibition halo (mm) after incubation, 24 and 48h, of the EAFFs (1mg/mL) and ampicilin (1mg/mL), used as standard drug.

- **Antinociceptive and antiedematogenic activities**

All experiments were conducted in quiet room at constant temperature (22°C). Male Swiss mice (25-30g) were pretreated with EAFFs (03, 04-08, 09-11, 12-22 and 22-29) from *K. brasiliensis* (100 mg/Kg, i.p.) intraperitoneally (i.p.) at a volume of 10mL/Kg. Control received vehicle (cremophor 4%, i.p.).

Acetic acid-induced writing. Male Swiss mice (25-30g) were used according to the method described by Koster et al. (1959). The total number of writings, following intraperitoneal administration of 0.6% acetic acid, was recorded for 20 min, starting 10 min after injection. The animals were pretreated 30 min before acetic acid administration.

Formalin test. Formalin-induced paw-licking was determined essentially as described by Hunskaar et al. (1985). The mice were pretreated 30 min before injection of formalin (1%, 20μL) into the subplantar area of the right hind paw. The duration of the paw licking, an index of nociception, was measured 0-5 min (first phase) and 20-25 min (second phase) after formalin administration. The antiedematogenic activity was evaluated 1-4h after injection of formalin. The volume (mL) of the paw was determined with a plethysmometer (Ugo Basile, Italy) before and after formalin. The edema was reported as the difference between the final and initial volume of the paw.

Statistical analysis

The results are presented as mean ± S.E.M. and were analysed by ANOVA, followed by Dunnett as the post hoc test. $P<0.05$ was used as the significance level.

Results and discussion

Among the EAFFs studied only EAFFs 09-11 (5mg/mL) demonstrated activity only against *S. aureus,* (inhibition halo: 0.7cm), while ampicilin (1mg/mL) were 1.2 cm.

EAFFs 03, 04-08; 09-11 and 22-29 from *K. brasiliensis* (100 mg/Kg, i.p.) significantly decreased the acetic acid-induced contractions (17.0±3.1; 4.4±0.8; 9.8±3.9 and 3.6±0.5 respectively) as compared to control (35.0±3.3). Table 1 show the results of the formalin test. The administration of EAFFs 22-29 (100 mg/kg, i.p.) reduced significantly ($P<0.05$) the licking time of the both phases, causing inhibitions of 44.7% in the 1st and of 91.5% in the 2nd phase. However, the others fractions (03, 04-08 and 9-11) inhibited only the 2nd phase of the nociception response.

Table 1.Efecct of the EAFF on the formalin-induced nociception in mice.

Group	Licking time (s)	
	1st phase	2nd phase
Control	59.0±4.4	28.2±4.2
EAFF 03	52.3±5.1	25.2±3.8
Fr 04-08	64.6±5.9	19.2±2.4
Fr 09-11	64.6±5.2	11.6±3.4*
Fr 22-29	32.6±3.2*	2.4±1.6*

Results are mean ± s.e.m. of the licking time. *=p<0.05 vs Control (ANOVA and Dunnett as the *post hoc* test).

The results show that EAFFs 04-08, 9-11 and 22-29 (100 mg/kg, i.p.) reduced significantly the paw edema induced by formalin after 1, 2 and 3 h, causing inhibitions ranging from 34 to 48%. However, the same dose of EAFF 03 did not significantly reduce the edema when compared with the control group (Figure 1).

The EAFFs from *K. brasiliensis* presented an antinociceptive effect, and inhibited the acetic acid writhings in mice that cause na acute inflammatory reaction related to the increase in the peritoneal fluid levels of PGE_2 and $PGF_{2\alpha}$ [7]. The EAFFs were also effective in the formalin test in mice, which involves 2 phases: neurogenic, with release of substance P, and inflammatory, with release of serotonin, histamine, bradikynin and prostaglandins [6, 8]. The EAFFs 04-08, 09-11 and 22-29 were also capable of reducing the edema in the formalin model of inflammation. Thus, the results of this study suggest that the antinociceptive effect of EAFFs are related to their antiinflammatory action.

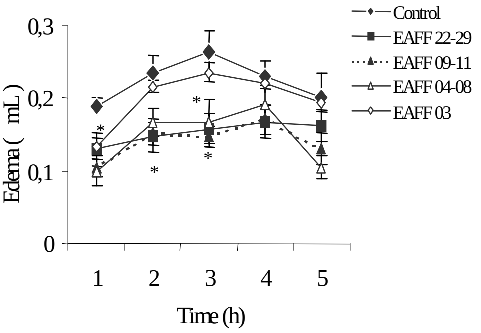

Figure 1. Effects of the EAFFs from *K. brasiliensis* in formalin-induced mice hindpaw edema. Each point represents mean ± s.e.m. indicated by vertical bars. *$P<0.05$ compared with control (ANOVA and Dunnett as the *post hoc* test).

Conclusion

Antinociceptive, antiedematogenic and antibacterial activities of the EAFFs from *K. brasiliensis* observed in this study contributed to elucidate the biological activity and its use in traditional medicine.

Acknowledgements
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BI-28 ANTIOXIDANT ACTIVITY OF QUINONES ISOLATED FROM *Auxemma oncocalyx* Taub

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Introduction

Auxemma oncocalyx Taub. ("pau branco") of the Boraginaceae family is found frequently in the State of Ceará, in the Northeast of Brazil. The stem bark of the tree is an astringent and popularly used in the treatment of wounds [2]. Previous pharmacological studies revealed that the alcoholic extract of the stem presents analgesic and anti-edematogenic [5], antiplatelet (Fontenele e Sousa) and antitumoral [7] activities. From the plant, a β-sitosterol glycoside and alantoin, responsible for some of its pharmacological properties, were isolated. Besides these coumpounds, at least six quinones were also isolated [8]. One of them (oncocalyxone A) is at least partially responsible for the biological activity of *A. oncocalyx*.

Previously we demonstrated the antiplatelet activity of this fraction [4]. The objective of the present work was to further explore the antioxidant activity of the quinone fraction from the heartwood of *A. oncocalyx*.

Experimental methods

- **Plant material and fractionation**

The plant was collected at the city of Pentecoste, Ceará, Brazil, and identified by Prof. A. G. Fernandes from the Biology Department. The plant voucher (n°. 18459) is deposited at the Prisco Bezerra Herbarium of the Federal University of Ceará. The quinone fraction (QF) was prepared from the grounded heartwood methanolic extract through aqueous extraction followed by lyophilization. Soluble constituents correspond to 80% of oncocalyxone A.

- **Antioxidant activity determination**

The *in vitro* antioxidant activity of the quinone fraction was evaluated according to the method proposed by Stocks et al. [9]. It is based on the inhibition of spontaneous lipid peroxidation of brain homogenates when incubated at controlled conditions. Both malondialdehyde (MDA) concentration and chemiluminescence (CL) emission were employed as parameters for lipid peroxidation evaluation. Adult male Wistar rats were anesthetized and sacrificed by cervical dislocation. The brains were perfused through the superior vena cava with ice-cold buffer solution pH 7.4, with the following composition (mM): 140 NaCl and 40 potassium phosphate, removed and homogenized with the same buffer (1:4 w/v). This homogenate was centrifuged for 15 min at 1000 g at 4°C. The supernatant was diluted three times with phosphate-saline buffer. Spontaneous brain lipid peroxidation was measured in 3 ml aliquots of the diluted homogenate transferred to 25 ml flasks for MDA measurement or 15 ml glass vials for CL studies. MDA was determined according to Fee and Teitelbaum [3] after one hour incubation at 37°C, in a shaker water bath. CL was measured in a Packard 1300R liquid scintillation analyser using the single photon monitor mode at 25°C as described by [6].

MDA and CL measurements were performed in the presence of different concentrations of QF dissolved in phosphate-saline buffer pH 7.4. The antioxidant capacity (AOC) is expressed as % as related to that of untreated brain homogenates. From the curves obtained from MDA and CL assays with different concentrations of the quinone fraction, $Q_{1/2}$ was calculated as the concentration able to inhibit 50 % of brain homogenate autoxidation.

Results and Discussion

In the present study the results showed that QF inhibited the lipid peroxidation of rat brain homogenates in a concentration-dependent manner. From the curve obtained from MDA (Fig. 1), the calculated value for $Q_{1/2}$ was 1.64 μg/ml. From the curve obtained from CL (Fig. 2), the calculated value for $Q_{1/2}$ was 2.06 μg/ml.. Barros et al. [1] demonstrated the inhibitory effect of α-tocoferol, an important natural antioxidant, on the lipid peroxidation in rat brain homogenates. The values of the $Q_{1/2}$ obtained for TBARS and QL for α-tocoferol were 14.40 and 10.90 mM (or 6.20 and 4.69 mg/ml). QF seems to be more potent than the α-tocoferol.

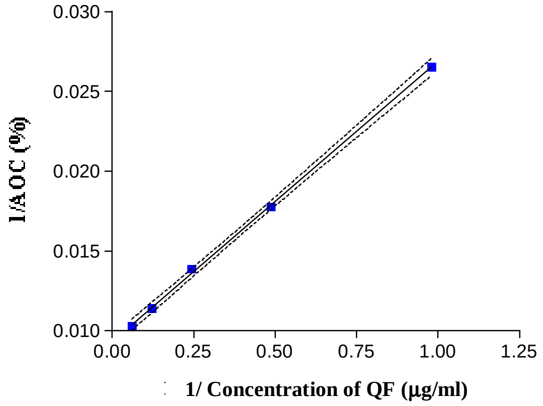

Figure 1- Antioxidant capacity (AOC) from different concentrations of the quinone fraction (QF) from ***Auxemma oncocalyx.*** Measurements were based on the inhibition of production of thiobarbituric acid reactive substances (TBARS). The curve was obtained from linear regression analysis.

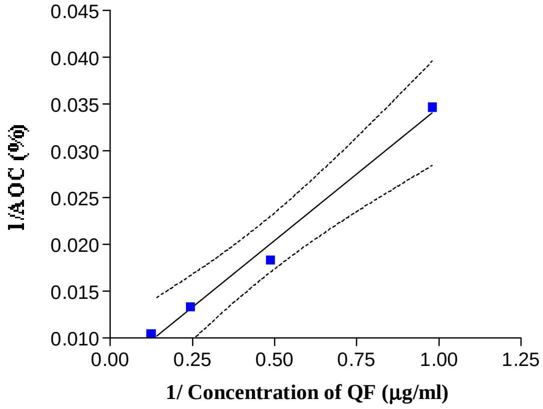

Figure 2- Antioxidant capacity (AOC) from different concentrations of the quinone fraction (QF) from ***Auxemma oncocalyx.*** Measurements were based on the inhibition of chemiluminescence emission. The curve was obtained by linear regression

Conclusion

The work showed that the quinone fraction from *A. oncocalyx* possess a potent antioxidant activity *in vitro*.

Acknowledgements

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BI-29 AN ALTERNATIVE COLORIMETRIC METHOD (MTT) TO MEASURE LECTIN-MEDIATED CELL PROLIFERATION

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Introduction

Lectins are proteins or glycoproteins of non-catalytic role and non-immune origin with at least two carbohydrate binding sites [2].

The lectin-mediated cell proliferation has been evaluated by a radioactive method involving ^{3}H-thymidine incorporation. A colorimetric assay has been largely applied to estimate the number of viable cells in microtitre plates which could perfectly replace the radioactive labelling used in most assays to measure cell concentration. This technique uses reduction of the tetrazolium salt, 3 – (4, 5 – dimethylthiazol – 2 – yl) – 2, 5 – diphenyl tetrazolium (MTT) inducing a MTT-formazan product [1, 3, 4]. However, the original colorimetric assay showed a variable background and an irregular cellular lost. The present investigation was undertaken to contribute to minimize the technical problems related to variable background and irregular cellular lost in the colorimetric assay.

Materials and methods

- **Cell culture**

Spleen cells from swiss albinic mice were adjusted to a concentration of 2x10^6 cells/ml in RPMI medium.

- **Colorimetric assay**

The MTT blank and cell culture samples were incubated at 37°C under 5% CO$_2$ for 4 h. After culture, the supernatants were removed and the residues were dissolved in 100 µl of 0.04N HCl in isopropanol (isopropanol-HCl), dimethyl sulfoxide (DMSO) and in 14 mM acetic acid in DMSO (DMSO-acetic acid). The resulting solutions were read in a spectrophotometer (Hitachi U-3200, Japan) and the absorbance was scanned between wavelengths of 350-700 nm.

- **Mitogenic activity of Con A.**

Aliquots (50 µl) of serial twofold dilutions of the lectin in RPMI medium, and 50 µl of cell suspension were added to each well. The absorbance was recorded in an ELISA plate reader at 492 nm. The mitogenic activity of the lectin was expressed as the following proliferation index (PI):

$$PI= \frac{A_L}{A_o}$$

Were, A_L means absorption of cultures with added lectin and A_o represents absorption of cultures without lectin [3].

Results

- **Evaluation of solvent efficiency to extract the MTT-formazan**

The results obtained through previously described assays were part of a comparative study of some solvents used to extract the MTT-formazan produced by cells on the colorimetric assay.

- **MTT-Blank**

There was a considerable absorption for isopropanol-HCl between 450 and 700 nm; DMSO exhibited a single maximum absorption at about 560 nm; for DMSO-acetic acid there was no absorption between 450 and 700 nm, the absorption was significantly reduced. All solvents showed a single maximum absorption at a wavelenght of about 390 nm.

Table 1: Maximum absorption of MTT-formazan produced by spleen cells, dissolved in isopropanol-HCl, DMSO and DMSO-acetic acid.

Solvent	*Maximum absorption (nm)*
Isopropanol-HCl	560
DMSO	560
DMSO-acetic acid	500

- **Cell culture**

Table 1 shows that for MTT-formazan dissolved in isopropanol-HCl and DMSO there was a single maximum absorption at about 560 nm. MTT-formazan dissolved in DMSO-acetic acid exhibited a new maximum absorption at about 500 nm. As expected, the change in absorbance was reflected in a color change.

- **Mitogenic activity of Con A.**

Con A was used to evaluate the modified colorimetric assay efficiency to measure the lectin-mediated cell proliferation. According to Figure 1, Con A showed a maximum mitogenic potential over spleen cells at concentrations of about 25 µg/ml.

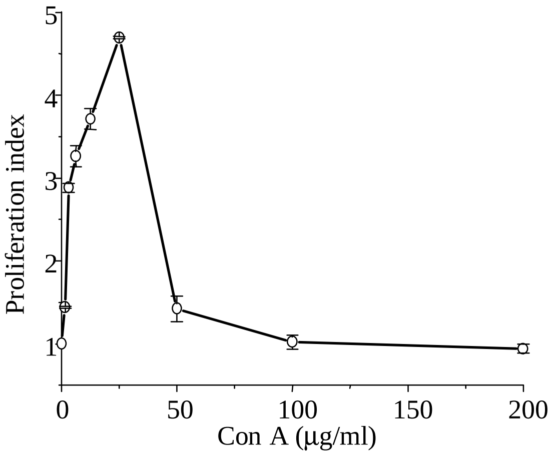

Figure 1: Mitogenic activity of Con A over spleen cells from swiss albinic mice.

Discussion

In the present work, three distinct solvents were evaluated to extract the MTT-formazan: isopropanol-HCl [3], DMSO, and DMSO-acetic acid. The DMSO-acetic acid was included in this study due to the use of DMSO to dissolve MTT-formazan in recent publications and its better solubility at low pH values [4].

The significant background observed when isopropanol was used as a solvent occurred probably due to its property to precipitate serum proteins at low pH values. MTT-blank in the use of DMSO as a solvent exhibited a single absorption at the wavelength at which MTT-formazan is known to peak; this was a indication that MTT residues present in MTT-blank could be converted to MTT-formazan when this solvent was used. These pitfalls in the colorimetric assay were reduced with acetic acid-DMSO to extract the MTT-formazan.

The pH drop in the extraction solvent by adding acetic acid to DMSO did not increase the wavelength corresponding to MTT maximum absorption. This fact supports the notion that formazan was not again converted to MTT under these conditions. It was previously observed that MTT-formazan is converted into MTT due to the drop in pH till below 4.0 [4].

The modified method revealed data already discussed by Kolberg and Sletten (1982), when using ^{3}H-thymidine incorporation to measure the Con A-mitogenic activity on human lymphocytes.

Conclusion

DMSO-acetic acid could be used to dissolve the MTT-formazan on a reproducible colorimetric assay. The colorimetric method evaluated was efficient to measure the lectin-mediated cell proliferation. Also, it was able to minimize technical problems related to irregular background or cellular lost.

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BI-30 EFFECT OF GLUCOSE/MANNOSE LECTINS IN LARYNX EPIDERMOIDE CARCINOMA CELLS (HEp – 2)

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Introduction

Lectins are proteins or glycoproteins that bind carbohydrates at cellular surfaces; these molecules have been applied in cancer studies. The acquirement of new chemistry therapeutics obtained by natural products with antitumour activity and less collateral effects have been important for cancer therapy. Camaratu bean (*Cratylia mollis*) is a natural forage from the Semi Arid Region of the State of Pernambuco, Brazil. *C. mollis* seed lectin (Cra) preparations (Cra Iso 1 and Cra Sephadex) were obtained. Concanavalin A, Con A, a lectin isolated from *Canavalia ensiformis* was utilized as an international pattern. Both lectins were obtained from the *Fabaceae* family. Con A and Cra are specific to glucose/mannose, have structural similarities but distinct *in vitro* binding properties [1]. The aim of this work was to study the effects on the proliferation and cellular morphology of two Cra preparations in larynx epidermoide carcinoma cells (HEp-2).

Experimental methods

- **Lectin preparations [2]**

C. mollis seeds (washed with distilled water and powdered) 0.15 M NaCl extract (4 °C, 16 h) was ammonium sulphate fractionated (F 40-60%) followed by affinity chromatography in Sephadex G-75 (Cra Sephadex, containing Cra Iso 1 and Cra Iso 4) and ion exchange chromatography in CM-cellulose (Cra Iso 1). Con A was obtained from Sigma Chemical Company Ltd., St. Louis, USA.

- **Immunological evaluation of Cra and Con A**

Cra Iso 1 (150 µg of protein) in 1.0 ml of sodium phosphate buffer, pH 6.2 in 0.15 M NaCl was emulsified in 1.0 ml of Freund's complete adjuvant and inoculated sub-cutaneously in a rabbit, followed by 6 inoculations (each 15 days) of Cra Iso 1 plus Freund's incomplete adjuvant. Anti-Cra Iso 1 serum production was evaluated by immunodiffusion assay carried out in 1% (w/v) agarose gel containing 0.1 M mannose using 10 µl of Cra Iso 1, Cra Sephadex, Con A and anti-Cra Iso 1 serum.

- **Cytotoxic assay**

The cellular proliferation was evaluated by the 3 - (4,5 – dimethylthiazol – 2 - yl) -2,5 – diphenyltetrazolium bromide (MTT) method. HEp-2 suspension of 5 x 10^4 cells (220 µl) were incubated with 22 µl of lectins (1.25, 2.5, 5.0 and 10.0 µg/ml) in a microplate and the results were obtained by the proliferation inhibition. Samples have activity when CI_{50} is equal or less than 4 µg/ml [3].

- **Cellular morphology**

The external membrane of the cell was evaluated by assays which verified the cellular morphology through the GIEMSA method.

Results and discussion

The immunodiffusion assay showed total identity between (Cra Iso 1 and Cra Iso 4) or partial identity (Con A) when anti-Cra Iso 1 serum was used (Figure 1).

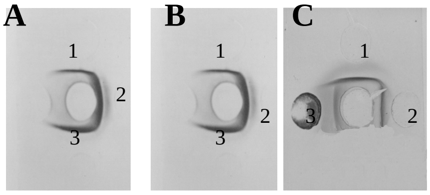

Figure 1 - Immunodiffusion in agarose gel

Center - Anti-Cra Iso 1 serum. **A -** 1, 2, 3, Cra Iso 1. **B -** 1, 2, 3, Cra Iso 4. **C -** 1, Cra Iso 1; 2, Cra Iso 4; 3, Con A.

Cra and Con A belongs to the same plant sub-tribe (*Phaseoleae)*, are specific to glucose/mannose carbohydrates and have similar subunits (31 kDa) [2]. The lectins are immunologically different and present distinct biological behaviours [1]. These results justify the use of more than one lectin with the same carbohydrate recognition to study the action of these proteins in biological investigations. The cytotoxic assay showed that Cra Iso 1 did not interfere in the HEp-2 proliferation. However Cra Sephadex and Con A showed inhibitory action when 10 μg/ml were used, without significant differences these results were not considered effective [3]. The cytotoxic assay with cells of different strains has been used to evaluate the toxicity of chemical and biological products. HEp-2 cells are well known and used to *in vitro* tests, including experimental cancerology. The negative results with the lectins make them potential tools to be used *in vivo*. The CI_{50} indicated the lectin dose to the morphological study. Some cellular colonies suggested cytostatic effect; the MTT assay did not show differences between cytotoxic or cytostatic effects, since it only evaluated metabolic active cells. Different results were obtained when Cra Sephadex, Con A and Cra Iso 1 were used for GIEMSA method (Figure 2); Con A and Cra Sephadex caused irreversible damages to the cells in the concentrations of 10 μg/ml. Cra Iso 1 did not show alterations in the cellular morphology.

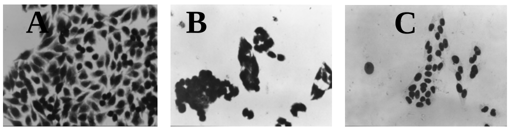

Figure 2 – HEp-2 cells treated with lectins and evaluated by the GIEMSA method

A - HEp-2 cells (Control, 100x); **B -** Con A (10 μg/ml, 100x); **C** – Cra Sephadex (10 μg/ml, 100x).

Conclusion

This study revealed that lectins with the same specificity can present immunological differences and distinct biological behaviors; Con A and Cra Sephadex are useful in the study of cellular morphology with HEp-2 cells.

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BI-31 ASSESSMENT OF MITOGENIC ACTIVITY OF FUCOIDAN ISOLATED FROM SEAWEED *Fucus vesiculosus*

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Introduction

Fucoidans or fucans are a family of sulfated homo and heteropolysaccharides composed mainly of α-(1→2) and α-(1→3) linked by L-fucose residues. The structures of these compounds vary among species and sometimes among different parts of the plant. In contrast to animal polysaccharides, the alga fucans contain besides neutral sugars, uronic acid and sulfated fucose [1]. These sulfated polysaccharides are involved in several biological activities including anti-inflammatory, antitumoral and mitogenic. We previously described the effect of fucans isolated from other seaweed species on cell proliferation, cell cycle and adhesiveness of both wild-type Chinese hamster ovary cells and xylosyltransferase-deficient mutant to several extracellular matrix proteins [2]. The objective of this work was to study *in vitro* mitogenic activity of fucans on human mononuclear cells.

Experimental methods

- **Materials**

Fucoidan isolated from *Fucus vesiculosus*; toluidine blue, concanavaline A, bovine serum albumin; α-(L)-fucose; D-galactose; L-xylose; glucuronic acid; D-mannose; isobutiric acid; tripan blue were purchased from Sigma Chemical Co. Tritiated thymidine purchased from NEM Life Science Products. Liquid scintillation from Ultima Gold DA Packard Instruments Co., Inc. Monoclonal antibodies and fluorescent substances, ficoeritrin (PE) and isotiocianate fluorescein (FICT) from Becton Dickinson Immunocytometry Systems. Cell culture wells purchased from Costar. Agarose from Bio Rad Laboratories. Acrylamide BHD Chemicals Ltd. (Poole, England).

- **Fractionation of fucans**

Fucoidan (Ft) from brown algae *Fucus vesiculosus* was fractionated with increasing volumes of acetone (1.0 v, 2.0 v and 3.0 v) yielding three fractions named F1, F2 and F3.

- **Electrophoresis**

Aliquot of each fractions (30μg) was applied in a 0.6% agarose gel in using different buffer conditions: 1.3 diaminopropane acetate 0.05 M, pH 9.0; barium acetate 0.04 M, pH 5.8; sodium phosphate 0.05 M, pH 8.0; sodium barbiturate 0.06 M, pH 8.6, KCl/HCl 0.03 M, pH 2.0. The compounds were then fixed in the gel in cetyltrimethylammonium bromide 0.01% for 4 h. The gel was dried and stained with 0.01% toluidine blue. Aliquots of the fractions (20μg) were applied in 6% polyacrylamide gel prepared with sodium barbiturate 0.06 M, pH 8.6 and stained with toluidine blue 0.01%.

- **Chemical analyses**

Fucose, xylose and uronic acids content of the polymer were estimated as described by Dische [3,4]. Total sugars were estimated by Dubois [5]. Sulfate was measured after hydrolysis (6N HCl, 100°C, 4h) by Dogson and Price [6] and protein was determined by Bradford method [7].

- **Mitogenic activity**

Peripheric blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque gradient. PBMC at 2 x 10^5 cells/well were suspended in RPMI 1640 supplemented with 10% AB + human serum and in presence of penicillin/streptomycin and cultured for 2 days, at 37°C, in 5% CO_2. Each condition was assayed in triplicates with or without 10 μg/ml fucoidans. (Ft, F1, F2 and F3). The proliferative response was measured by $[H^3]$-tymidine incorporation [14]. Cells were pulsed with 1μCi of $[H^3]$-thymidine per well for the final 18 h of culture. Tymidine uptake was quantified after cell harvester in a scintillation counter and proliferative response expressed as stimulation index (SI) [SI = $CPM_{\text{cells with mitogens}} - CPM_{\text{cells without mitogens}} / CPM_{\text{cells without mitogens}}$]. The type of cell type stimulated by the fucoidans was determined by flow cytometry using a panel of monoclonal antibodies. The mononuclear cells were obtained according to procedures described above [9]. Aliquots of 1000 μl of cells suspension (1 x 10^6 cells/ml) were stimulated by 50μg of each fucan fractions. Control cells were maintained in media alone. Cells were sorted after 48 hours of incubation [15].

Results and discussion

Fucoidan fractions had an average molecular weight of 120,000 D; similar to other fucoidans previously described. They are high disperse in agarose gel and are composed mainly of L-fucose-sulfate. The chemical analyses showed that fraction F1 have a high content sulfate in relation F2.

Study of the biological activities using human PBMC showed that fraction F1 and Ft have mitogenic activity similar to Concanavalin A at the concentration 20 and 50 µg/ml (Figure 1). This is a very interesting finding to further pursue once those seaweed are quite abundant and free of contaminant as HIV or other virus. In addition, some of these fucans have shown anti retroviral activity.

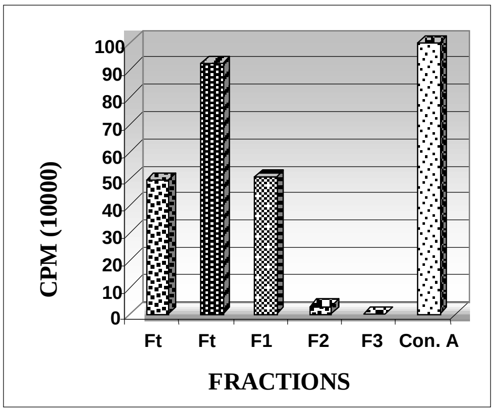

Figure 1 – Cell proliferation in response to fucoidan stimulation

The flow cytometry showed that 45% of the cells stimulated by F1 fraction were TCD4$^+$ cells type (Table 1).

Table 1 – Activity of fucoidan fractions upon human PBMC as determined by cell sorting.

Marker	Control %	Ft %	F1 %	F2 %
CD8	50	46	33	43
CD4	35	47	45	30
CD19	6	8	8	2
CD3	86	76	70	75
CD22	6	10	8	5
CD5	80	81	62	69
CD7	84	88	73	83

Conclusions

This work has shown that the fraction F1 when incubated with PBMC induced the cellular proliferation at a concentration of 20µg/ml. Cell sorting showed proliferation of CD4 (45%).

Acknowledgements

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BI-32 EFFECTS OF *Copaifera langsdorffii* OLEO-RESIN AND THE ISOLATED CONSTITUENT KAURENOIC ACID ON ACETIC ACID-INDUCED COLITIS IN RATS

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Introduction and Goals

Copaifera langsdorffii Desf. (Leguminaceae), popularly know as "Copaíba", is a large tree that grows abundantly in Amazonas, Pará and Ceará states of Brazil. The oleo-resin (Copaíba balsam) obtained from its stem bark is a reputed folk remedy in its natural form for the treatment of sore throat, urinary and pulmonary affections, to promote healing of skin ulcers and wounds [2, 10]. Phytochemical studies carried out on oleo-resin of *C. langsdorffii* revealed the presence of essencial oils (8%; β-caryophyllene, caryophyllene oxide, β-elemene, α-cis-bergamotene, ar-curcumene, and α-trans-bergamotene), and a mixture of diterpenes (70%; kaur-16-en-19-oic and polyalthic acids) [5]. Previous studies established the anti-inflammatory effect of ORCL and antinociceptive, antimicrobial and smooth muscle relaxant effects of kaurenoic acid [1,4,6,8,11,13]. The present study analysed their anti-inflammatory potential in acetic acid-induced animal model of rat colitis that simulate inflammatory bowel disease in humans.

Methodology

The oleo-resin of *Copaifera langsdorffii* (ORCL) was employed at intrarectal doses of 200 and 400mg/kg as an aqueous suspension using the vehicle (2% v/v Tween 80 in distilled water). These doses were selected based on preliminary experiments that showed a significant protection against ethanol induced gastric damage (PAIVA *et al.*, 1998). Isolated constituent kaurenoic acid was employed at rectal doses of 50 and 100mg/kg body weight as an aqueous suspension using the vehicle (5% v/v DMSO in distilled water). The test drugs or vehicle (controls) were administered rectally in a volume of 10mL/kg.

- **Colitis Induction**

Colonic inflammation was induced in rats following the method of MASCOLO *et al.* [7]. Rats were fasted for 36 h and anaesthetized with ether. A rubber cannula (8 cm long) was inserted into the colon, via the anus and a 2 mL solution of 4% acetic acid was instilled into the lumen of the colon. The rats were then allowed to recover from the anaesthetic and returned to their cages. Twenty four hours later, the animals were sacrificed and analysed for macroscopic lesions, colonic wet weights and for the content of thiobarbituric acid-reactive substances (TBARS).

- **Assessment of Macroscopic Injury**

The colons were excised, opened down their mesenteric borders, and cleansed of luminal contents. Severity of gross macroscopic injury was graded according to the following score system [3]: 0, normal appearance; 1, focal hyperaemia, no ulcers, single site of ulceration without associated inflammation; 3, ulceration with inflammation at one site; 4, two or more sites of discrete ulceration and inflammation; 5, major site of injury or inflammation extending 1-2 cm along length of colon; 6-10, score increased by one for each additional centimetre of damage or injury beyond 2 cm.

- **Assessment of the wet weight**

The wet weight of the colon was assessed as described by MASCOLO *et al*. [7]. A 5-cm segment of the distal colon, 3 cm proximal to the anus was resected, the lumen rinsed with ice-cold saline and weighed.

- **Determination of Malonaldehyde (MDA)**

The contents of TBARS (μmol MDA/g of tissue) was measured by the method of UCHIYAMA & MIHARA, 1978, to assess lipid peroxidation. Colon removed and homogenized with cold 1.15% KCl to make a 10% homogenate. To a 0.5 mL of 10% homogenized pipetted into a 10 mL centrifuging tube were added 3.0 mL of H_3PO_4 1% and 1.0 mL of thiobarbituric acid (0.6%) aqueous solution. The tubes were heated for 45 min in a boiling water-bath and the reaction mixture was then cooled in an ice bath followed by the addition of 4.0 mL of n-butanol. The contents were mixed for 40 s with a vortex mixer, centrifuged at 1200 g for 10 min, and the absorbance of the organic layer was measured at 520 and 535 nm. The results were expressed by µmol MDA g^{-1} of tissue.

Results

The obtained results are presented in Tables 1 and 2 below.

Table 1. Effects of *Copaifera langsdorffii* oleo-resin (ORCL) and kaurenoic acid (AK) in rats on acetic acid colitis.

Treatment	Lesion Score	Colon Wet Weight
Control vehicle	0±0	336.38±12.76
Control acetic acid	4.57±0.20***^{a}	767.85±43.21***^{a}
ORCL 200mg/kg	3.60±0.24	615.40±68.18*^{b}
ORCL 400mg/kg	2.50±0.57***^{b}	635.87±35.20*^{b}
AK 50mg/kg	4.33±0.33	651.00±34.49
AK 100mg/kg	4.60±0.25	506.20±16.40***^{b}

^{a}vs control vehicle, ^{b}vs control acetic acid, *p<0.05, ***p<0.001. ANOVA and Mann Whitney Test.

Table 2. Effects of *Copaifera langsdorffii* oleo-resin (ORCL) and kaurenoic acid (AK) on malonaldehyde levels of in rats on acetic acid colitis.

Treatment	µmol MDA/g of tissue
Control vehicle	1.32±0.54
Control acetic acid	8.45±1.32***^{a}
ORCL 200mg/kg	1.18±0.54**^{b}
ORCL 400mg/kg	3.61±0.60*^{b}
AK 50mg/kg	1.11±0.19**^{b}
AK 100mg/kg	2.13±0.98**^{b}

^{a}vs control vehicle, ^{b}vs control acetic acid, *p<0.05, **p<0.01. ANOVA and Mann Whitney Test.

Conclusion

Both ORCL and the isolated constituent kaurenoic acid were able to effectively reduce the inflammatory process associated with the acetic acid colitis as evidenced by decreases of colonic tissue weights, the lesion score as well as the lipoperoxidation product malondialdehyde. The study substantiates the popular use of oleo resin in inflammatory disease conditions and merits further investigation on their mechanisms.

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BI-33 DETECTION OF THE ANTIBIOTIC ACTIVITY IN FRESH AND DRY PLANTS OF *PLECTRANTHUS BARBATUS* ANDR

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Introduction

Plectranthus barbatus Andr. it is a plant probably originating in the African continent, which is found in Brazil from Rio Grande do Sul to Amazonas.

Forskolin is a diterpene isolated from the roots of that plant. It has been referred as being positive inotropic, antihypertensive, potent estimulator for adenilate ciclase from many tissues and platelet aggregation inhibitor [1]. Extracts of *Plectranthus barbatus* have been used in folk medicine of many countries in treatment of several disorders cardiovascular, breathing, gastrointestinal and of the central nervous system.

The objective of this study was to detect the antimicrobial activity from extracts of root, stem and leaves of *P. barbatus*, obtained by dry and fresh plants (recently collected).

Experimental methods

• Materials

The plants were collected in the Company of Provisioning and Rural Extension of the State of Pernambuco (EBAPE), where they have been cultivated for herbal remedies production.

Acetone, ethanol and methanol were the solvents used in the preparation of the extracts.

The tested micro-organisms were: *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus anthracis*, *Bacillus mycoides*, *Enterococcus faecalis*, *Mycobacterium smegmatis*, *Micrococcus luteus*, and *Streptococcus pyogenes*.

Culture-test were maintained in the media Mueller Hinton agar, GL (Glucose + extract of yeast agar) and agar blood.

▪ Preparation of the extracts

Root, stem and leaves extracts were prepared in the proportion of 20mL of solvent (acetone, ethanol and methanol) for 8g of material. The extractions were made by maceration at rest for eight days in room temperature and for agitation during 4:00 (≈200rpm) in controlled temperature of 28 - 30 °C.

The total volume of each extract was filtered and evaporated to the dryness; starting from the dry extracts, the solutions were prepared to the concentration of 30.000 µg/mL in DMSO.

▪ Antimicrobial activity

The antimicrobial activity was verified "in vitro", for the conventional method of diffusion in paper disk [2]; the tests were accomplished in three repetitions and the results were appraised for the average of the diameters of the halos of inhibition of the bacterial growth, in the three repetitions.

Petri dishes containing the medium of cultivation, they were sowed with the suspension of the micro-organism under test. The bacterial suspensions were prepared in saline solution 0.89%, starting from recent cultures and standardized to an optical density from 0.2 of absorbance to 600nm, equivalent to the turvation 0.5 of the scale of McFarland [3], that it corresponds to concentration of approximately 10^7 UFC/mL. For the sows, 0.1 ml of the microbial suspension was put onto surface of the medium and spread evenly with loop of Drigalsky. Onto medium inoculate, discs of paper syrup soaked with 20µl of the solution of the extract were put, being each disk with a concentration of 600 µg of the crude extract. After the placement of the discs, the plates were maintained in refrigerator (≈12 °C) for 30 minutes, for active substance diffusion beginning in the medium before initiate the growth of the micro-organism. After the diffusion period, the plates were incubated during 24:00 h to 35ºC. The mean diameter of the halos from microbial growth inhibition was evaluated in three repetitions for each microorganism and treatment (fresh and dry plants).

Results and discussion

It was verified that the cetonic extract of softened dry leaves, presented the best activity against all of the micro-organisms, while the cetonic extract of the fresh leaves didn't present any activity against none of the tested micro-organisms (Table 1).

The ethanolic extract of dry leaves, obtained by agitation, it was more active against *Enterococcus faecalis*, with the same activity that presented by the extract of dry leaves macerated in acetone, against *Streptococcus pyogenes* and *Mycobacterium smegmatis.*

Table 1 - Antimicrobial activity of root, stem and leaves extracts of *Plectranthus barbatus* Andr. obtained by dry plants for maceration

	Dry Plants - halos (mm)								*Fresh Plants - halos (mm)*							
	Microorganisms								**Microorganisms**							
EXTR	**B.a**	**S.a**	**B.s**	**E.f**	**B.m**	**S.p**	**M.s**	**M.l**	**B.a**	**S.a**	**B.s**	**E.f**	**B.m**	**S.p**	**M.s**	**M.l**
RA	12	13	13	13	12	10	12	20	10	-	10	12	9	-	-	14
RE	10	10	11	10	8	-	11	15	10	-	10	10	9	-	12	14
RM	15	14	15	14	14	10	14	18	11	12	12	12	12	-	14	15
CA	13	14	13	12	12	14	11	20	10	13	12	10	9	14	13	17
CE	13	14	13	11	12	13	15	20	11	11	12	-	9	-	14	13
CM	11	11	11	10	11	9	15	17	13	10	12	10	11	-	14	15
FA	17	17	18	15	17	15	16	20	-	-	-	-	-	-	-	-
FE	11	-	10	-	8	-	-	-	8	-	9	12	9	12	-	9
FM	16	11	15	13	14	10	-	12	14	-	10	-	8	10	-	10
Máx.	17	17	18	15	17	15	16	20	14	13	12	12	12	14	14	17

Microorganisms: B.a - *Bacillus anthracis;* S.a - *Staphylococcus aureus;* B.s. - *Bacillus subtilis*; E.f. - *Enterococcus faecalis*; B.m - *Bacillus mycoides*; S. p - *Streptococcus pyogenes*; M. s - *Mycobacterium smegmatis* ; M. l - *Micrococcus luteus*. EXTR - Extracts. RA, RE, RM - root acetone, ethanol and methanol; CA, CE, CM - stem acetone, ethanol and methanol; FA, FE, FM - leaf acetone, ethanol and methanol; Máx - Maximum value

However, when prepared starting from fresh leaves, no activity was observed in these extracts, against any of the tested micro-organisms. *Micrococcus luteus* was more sensitive to extracts of dry roots in acetone, obtained by agitation (Table 2).

Table 2 - Antimicrobial activity of root, stem and leaves extracts of *Plectranthus barbatus* Andr. obtained by dry and fresh plants for agitation

	Dry Plants - halos (mm)								*Fresh Plants - halos (mm)*							
	Microorganisms								**Microorganisms**							
EXTR	**B.a**	**S.a**	**B.s**	**E.f**	**B.m**	**S.p**	**M.s**	**M.l**	**B.a**	**S.a**	**B.s**	**E.f**	**B.m**	**S.p**	**M.s**	**M.l**
RA	13	13	12	12	10	9	16	23	-	-	8	-	-	-	9	10
RE	11	13	13	15	12	-	13	23	8	9	9	9	8	-	-	14
RM	11	13	12	13	11	-	13	19	8	-	8	-	8	-	8	8
CA	11	12	12	-	9	-	12	16	8	11	10	8	9	8	10	12
CE	10	10	11	10	8	-	10	20	8	9	8	-	-	-	-	9
CM	11	10	12	9	10	-	12	15	8	9	8	-	-	-	9	10
FA	11	9	12	13	16	8	12	15	12	10	11	-	13	10	-	15
FE	15	13	15	20	16	15	15	20	-	-	-	-	-	-	-	-
FM	16	11	13	12	14	14	12	15	11	-	-	-	-	9	-	-
Máx.	16	13	15	20	16	15	16	23	12	11	11	9	13	10	10	15

Microorganisms: B.a - *Bacillus anthracis;* S.a - *Staphylococcus aureus;* B.s. - *Bacillus subtilis*; E.f. - *Enterococcus faecalis*; B.m - *Bacillus mycoides*; S. p - *Streptococcus pyogenes*; M. s - *Mycobacterium smegmatis* ; M. l - *Micrococcus luteus*. EXTR - Extracts. RA, RE, RM - root acetone, ethanol and methanol; CA, CE, CM - stem acetone, ethanol and methanol; FA, FE, FM - leaf acetone, ethanol and methanol; Máx - Maximum value

It was not allowed to carry out a comparative study due to the absence of reference compound for crude extracts of *P. barbatus*.

Conclusion

All the micro-organisms were sensitive to the extracts of root, stem and leaves of *Plectranthus barbatus*. The study showed that extracts of dry plants are more active than extracts of fresh plants. The absence of activity in some extracts from fresh plant suggests that the presence of water in the plant interferes with the extraction of the active substances, reducing or even vanishing its activity.

Acknowledgements

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PP-01 DENSIFICATION OF PLANT EXTRACTS BY SLUGGING AND ROLLER COMPACTION

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Introduction

The modern technology of tablet formulation and processing by direct compression requires constituents exhibiting flowability and compressibility. Dry powdered plant extracts do not show these properties needed to be processed by direct compression. Additionally, high doses of dried extracts are in many cases required. To solve these problems, dry granulation is the technique of choice [1].

Dry granulation by slugging or roller compaction has been a usual process to form granules of materials, which are sensitive to moisture or heat. In addition flowability and compressibility is improved. Dry granulation can be achieved by either forming slugs, using a tablet press, or processing the powder using a roller compactor. The desired particle size is then adjusted by milling and sieving. Mechanical properties of granules are important for a subsequent process [2, 3].

The objective of the present was to evaluate the physical and mechanical properties of spray dried extracts from *M. ilicifolia* granules prepared by slugging and roller compaction.

Experimental

- **Materials**

Spray dried extract from *M. ilicifolia* was produced using a NIRO ATOMIZER spray drier. It was blended with Aerosil 200® (Degussa, Germany) 1.5 %, and magnesium stearate (Bärlocher, Germany) 2.8 %.

- **Compaction**

The mixture was blended in the Turbula® mixer (Willy A. Bachofen, Switzerland) for 5minutes.

Slugging: Briquettes of 0,8 g were produced on a single punch tablet press EK 0 (Korsch, Germany) at compression force of 22.0 ± 1.0 kN using flat faced tooling of 17 mm in diameter.

Roller compaction: The powder compacts were prepared using a roller compactor (GMP-Mini-Pactor®, Gerteis, Switzerland) at 5, 10 and 15 kN/cm, with gap size of 1 mm (automatically adjusted).

- **Granulation**

The milling conditions were kept constant for all compacts. They were milled in a dry granulator (Erweka TG IIS with motor Erweka AR 400, Germany) to obtain granules with a particle size below 2.00 mm. Then the material was processed through an oscillating granulator (Erweka FGS with motor Erweka AR 400, Germany) until all product was passed through a 1.0 mm sieve.

- **Particle size analysis**

The particle size distribution of the spray dried extract was determined using the laser diffraction analyzer Helos KA (Sympatec, Germany). The particle size distributions of the granulations were determined by sieve analysis. 50 g from each granulate batch were tested (roller compacted and slugging).

- **Bulk and tapped density, Hausner´s ratio and Carr´s compressibility index**

The density parameters were determined using 10,0 g of each material in a 50 ml graduated cylinder (n = 3) (tapping device: J. Engelsmann, Germany).

- **Evaluation of the compressibility [4]**

Compacts were produced at various pressures (between 20 and 110 MPa) on a single punch tablet machine using flat faced tooling of 10 mm in diameter (n = 3). Height, diameter and weight were determinated to obtain a calculated density. This value was divided by the true density (evaluated by a Beckmann air comparison pycnometer model 930, USA), hence the relative density (D) of every sample was obtained. Then the Heckel equation (eqn. 1) was applied to plot the compaction response diagrams. The mean yield pressure (Py) was calculated as 1/K.

$$\text{Equation 1: } \ln\left(\frac{1}{1-D}\right) = KP + B$$

Where: D is the relative density of the compact at pressure P, K is the slope, and B is a function of the initial bulk volume.

- **Influence of the granulation method on the tensile strength [5].**

Compacts were produced in between 40 and 240 MPa on a single punch tablet press using flat face tooling of 10 mm diameter (n = 6). The crushing strength was determined 24 h after production and the tensile strength was calculated (eqn.2):

$$\text{Equation 2: } TS = \frac{2.F}{\pi.d.h}$$

Where: TS is the tensile strength, F is the crushing force, d is the compact diameter, and h is the compact thickness.

Results

- **Particle distribution**

The mean particle size of the pure extract increased from X_{50} = 22.4 µm to X_{50} = 600 µm of the granules, independent of the applied compaction method

- **Materials Properties**

The mechanical properties of the mixture were enhanced by the granulation. The bulk and tapped density of the granules obtained from roller compaction showed a slight increase with increasing roller compression force (table 1). The slugging granules did not differ from the other granules.

Table 1. Pharmaceutical Properties of the materials

Material	bd^1	td^2	H^3	CI^4
Mixture	0.500	0.690	1.376	27.50
Slug	0.659	0.706	1.071	6.59
Roller 5 kN	0.645	0.714	1.107	9.68
Roller 10 kN	0.690	0.741	1.074	6.90
Roller 15 kN	0.741	0.800	1.080	7.41

^{1}bulk density; ^{2}tapped density; ^{3}Hausner´s ratio; ^{4}Carr´s Compressibility index.

The Heckel plot (figure 2) showed a linear compression section indicating good plastic deformation. This was confirmed by good correlation coefficients (table 2).

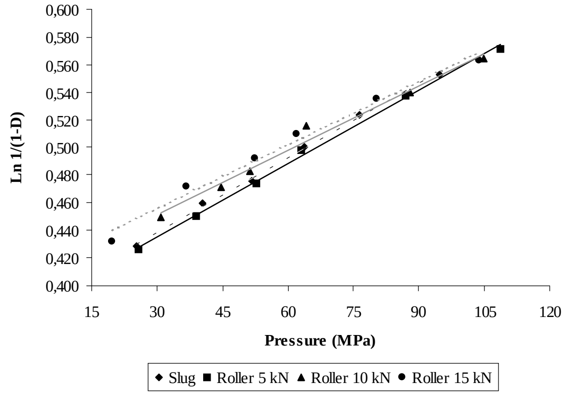

Figure 2. Heckel plot for the granules

Granules from slugging and roller compaction at 5 kN/cm showed similar properties (K, B and Py) while the denser granules (roller compaction at 10 and 15 kN/cm) showed a higher resistent against deformation and denser appearence in the die (B) (table 2).

Table 2. Parameters for the Heckel model

Param.	Slug	Roller 5 kN	Roller 10 kN	Roller 15 kN
K	0.0018	0.0018	0.0016	0.0015
B	0.3843	0.3823	0.4041	0.41
Py	555.56	555.56	625.00	666.67
R^2	0.9983	0.9976	0.9845	0.9849

Figure 3 shows the change of the compact porosity (**ε**) and the tensile strength with increasing compaction pressure. It was found that, pre-densification by slugging or roller compaction is decreasing the compacts tensile strength. However, the tensile strength profiles of granules prepared by slugging and roller compaction at 5 and 10 kN/cm were very similar. At 15 kN/cm the roller compaction granules showed a decrease in tensile strength. This is a result of work hardening and the production of granules that tended to show increasing resistance to deformation upon recompression.

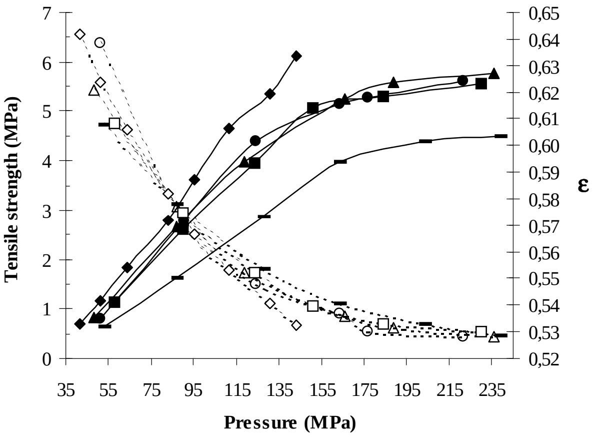

Figure 3. Compacts strength versus compression pressure. (Full lines tensile strength, open lines porosity: ◆ mixture, ■ Slug, ▲ Roller 5 kN, ● Roller 10 kN, - Roller 15 kN)

Conclusions

The compaction of *M. ilicifolia* spray dried extracts to obtain granules can be performed by slugging or roller compaction. However, the process of roller compaction is much faster and cost effective.

The mean particle size is independent from the compaction method and the compression force.

The higher the densification during the granulation process the higher the yield pressure during tableting.

The more plastic deformation occurred during granulation the less plastic deformation remains for tableting, hence the tensile strength decreases.

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PP-02 HYDROXYPROPYLMETHYLCELLULOSE-CARBOXYPOLYMER MATRIX TABLETS FOR THE CONTROLLED RELEASE OF SODIUM DICLOFENAC: FORMULATION AND *IN VITRO* CHARACTERIZATION

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Introduction

Over the last two decades extensive research has been performed in the design and development of sustained release systems.

Hydrophilic matrix tablets for the sustained drug release, contain a therapeutic agent dispersed in a compressed water-swellable polymeric core [1].

In recent years hydrophilic cellulose derivatives have become the most widely used polymers for novel drug delivery systems.

One of the most popular hydrosoluble polymers used in controlled release dosage forms is hydroxypropylmethylcellulose (HPMC) [2]. On the other hand, carboxypolymer (Carbopol 934) is a high molecular weight, cross-linked polymer of acrylic acid co-polymerized with polyalkyl-sucrose. The final product contains many carboxylic acid groups. The hydrogel presents high viscosity characteristics being influenced by pH changes and presence of electrolytes [3].

Diclofenac Sodium was selected as a model drug in the formulation of sustained release HPMC-carbopol matrix tablet.

Objectives

The purpose of this study was to formulate different controlled release matrix tablets, and to evaluate the drug release characteristics as a function of polymeric swelling behavior, influences of pH, and the polymer concentration.

Experimental

The selected drug was Diclofenac Sodium, hydroxypropylcellulose and carbopol were used as hydrophilic polymeric matrix formers. The disintegrant was starch, the filler was lactose and magnesium stearate was used as the lubricant. The carriers HPMC/carbopol (10%, 20% and 30%) at different ratios (1:1, 3:1, 1:3) were blended with the drug and the others excipients in a planetary mixer for 5 minutes. Thereafter, the powders were granulated with 10% w/v PVP EtOH solution, sieved using a 1.40 mm mesh screen, and the obtained granules were dried in a hot air oven at 40°C for 3 hours. Finally, the granules were dried and sieved using 1.00 mm mesh before tableting. Tablets of approximately 280 mg weight each were prepared from these granules after addition of starch and magnesium stearate. Tablets were compressed using a single punch-tableting machine Erweka, Heusenstamm-Germany, with 9 mm flat round punches. A constant compression force was obtained by using the same distance between the upper and lower punches. Each tablet contained 50 mg of Diclofenac Sodium. Three batches were prepared for each formulation.

In Vitro Drug Dissolution Studies

Drug release profiles of the tablets were evaluated in vitro using a dissolution test apparatus (Hanson Research, SR8 8-Flask Bath). The USP paddle method was selected to perform the dissolution profiles of Diclofenac Sodium.

The same test for all the formulations was carried out in 900 mL 0.1 N HCl, and phosphate buffer, pH=6.8 (USP XXIV) maintained at 37 ± 0.5^{o}C at a paddle rotation speed of 50 rpm.

Results and discussion

The weight deviation of the nine matrix tablets was acceptable due to the granule flowability properties. The minimal friability obtained confirmed the suitability of the wet-granulation technology for the preparation of these HPMC-carbopol matrices. It is known that the release rate of drugs from hydrophilic matrices containing HPMC-carbopol, is affected by pH changing. Thus, the dissolution rate was investigated at pH 1.2 and 6.8. For all the formulations, the release rate of Diclofenac was extremely low at acidic pH, since after 2 hr only less than 1% of drug was released, spectrophotometrically observed. Thus independent of pH the total amount of polymer is the main factor witch controls the dissolution rates of diclofenac.

These rates increase as the amount of total polymer increase too.

Figure 1: HPMC/carbopol (10%) at different ratios (3:1, 1:1, 1:3)

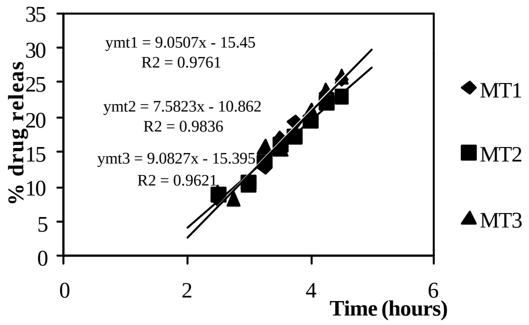


Figure 2: HPMC/carbopol (20%) at different ratios (3:1, 1:1, 1:3)

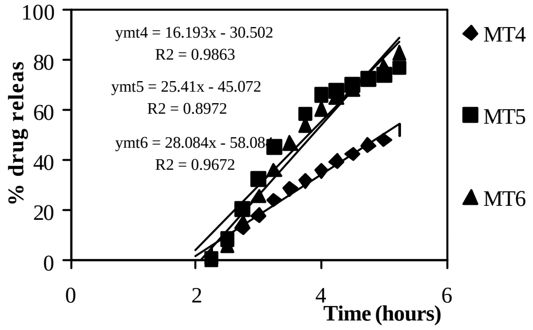
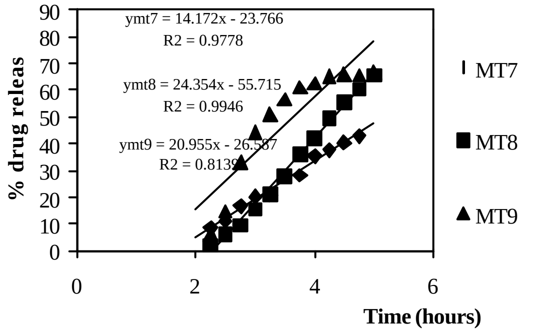


Figure 3: HPMC/carbopol (10%) at different ratios (3:1, 1:1, 1:3)



Conclusion

Sodium Diclofenac controlled-release matrices were prepared successfully by wet granulation utilizing HPMC/carbopol as carriers. Results demonstrated the suitability of these polymeric matrices for a sustained-release rate, due to a zero-order kinetic over 6 hr. Compared to conventional tablets, release of diclofenac from these controlled-release HPMC/carbopol matrices was prolonged. Thus, an oral controlled-release dosage form to avoid gastric or duodenal irritation was accomplished.

Acknowledgements

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PP-03 TECHNOLOGICAL DEVELOPMENT AND COMPARATIVE LIODISPONIBILITY OF TABLETS OF CALCIUM SUPPLEMENT MADE OF OYSTER SHELL POWDER

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Introduction

Osteoporosis is a multifactorial syndrome characterized by progressive loss of bony mass, with countless known causes and countless risk factors for its appearance. It constitutes a great problem of public health. According to the Brazilian Society of Osteoporosis, between 35 and 52% of Brazilian women who are over 50 years of age will have osteoporosis until the year 2000 [1]. Experiments made with several animal species showed clearly the influence of calcium ingestion in the swinging of the bony mass [2-7]. We presented the development of tablets obtained from the power of oyster shells collected in the coastline of Recife, nowadays disposable. The calcium dosage was accomplished through photometric flame (Eq.Mod/fab) and through spectrophotometry of atomic absorption (Mod/fab). Determinations of the quality parameters of those tablets and comparisons with existent tablets in the market were made. The comparative liodisponibilty followed the methodology of dissolution presented in USP XXIII. The objective of this work was the study of the liodisponibility of tablets of calcium carbonate developed from the powder of oyster shells and the comparison with a reference medication.

Experimental Methods

• Materials

Analytic scale (Teclabor ltda., mod-AC200); dissolutest (Ética eq. Científica S/A, mod-299.1), tablets hardness test (Off Tec Galileu, série-9404), friabilator (Ética eq. Científica S/A, mod-300), mixer in "V" (Alwis, no. 849493), eccentric compressor (Neuberger, mod-3135N), oscillating granuler (Lawes, no. 00032598), flame spectrophotometer, spectrophotometer of atomic absorption, talc explocel, polivinilpirrolidona, oyster shell powder, cellulose microcrystalline and reference tablets.

• Obtaining of the tablets

The tablets were obtained by the granulation process through humid way being used as wetting liquid a hydro alcoholic solution of PVP. A machine of eccentric compression was used with oblong puncture. The formula was defined for tests with weight of 500.00g as exhibited in the chart 1:

Table 1. Oyster Shell Powder Formula.

FORMULA	%
Oyster shell powder	80.64 %
Celulose microcristalina	9.33 %
Talco	2.50 %
Explocel	2.93 %
PVP	4.60 %

For the studied tests, we started from standardized assay made within the following conditions:

Mixture of the components: 3 minutes using a mixer type in "V" with 2.0kg of capacity;
Wetting process: 5 minutes with 400 ml of the ligante solution of alcoholic PVP in a planetary whisk;
Granulation: in rocking granuler using a 2.0mm mesh and after drying, calibration using a 1.0mm mesh;
Drying : done in 1.0 hour in stove with exhaustion in a 45° C temperature.

• Physico-chemical assays of he tablets

Assay of medium weight, hardness, friability and content were accomplished according to USP-XXIII.

• Dosage of the tablets

Analysis of the calcium content in the tablets was made according to Brazilian Pharmacopoeia (2nd edition) being used the titration method.

- **Dissolution**

The dissolution was made in acid and using a dissolutest following the USP Methodology. 5ml of samples of the dissolution were collected in 30, 60, 90. The collecting was made using syringes with filters.

- **Calcium dosage in the dissolution**

The dosage of calcium was made through both flame photometry and spectrophotometry of atomic absorption. Solutions of standard calcium of well-known concentrations were used for construction of the curve of calibration of the equipment and for the identification of concentrations of the samples and, through that, calculating the profile of dissolution of the tablets during the time.

Results and discussion

The results of the physico-chemical analyses are shown in the chart 2. The content weight of the calcium in the analyzed tablets is 500mg to 1,500mg of the total mass.

Table 2. Physico-Chemical properties of tablets.

	Test	**Reference**
MEDIUM WEIGTH	1.55g	1.59g
FRIABILITY	0.38 %	*
DISINTEGRATION	< 5min	10min
HARDNESS	15.0 Kgf	9.58 Kgf
ASSAY	96.00 %	98.40 %

We can verify that it is necessary a small adjustment in the content in the formula test. We also observed that in the disintegration we obtained a better result than in the compressed reference. Brennan *et al.* 1991, concluded in one of his researches that the breakup of the tablets of calcium supplement is an important indicator of the biodisponibility of that ion and that the time of disintegration is decisive for the dissolution of those tablets [8]. We also observed that the reached hardness is compatible with the posterior stage of coating of the tablet. We opted for the non coating, because the active principium does not demand and the coating absence increases the economic viability of the product. Related to the dissolution we can see in figure 1 very close profiles, what assures us, with support in the physical-chemical data, that the product test has characteristics of interchangeable liodisponibility. We also showed in the graph the specified limit in USP – XXIII that is minimum of 75% in 30 minutes.

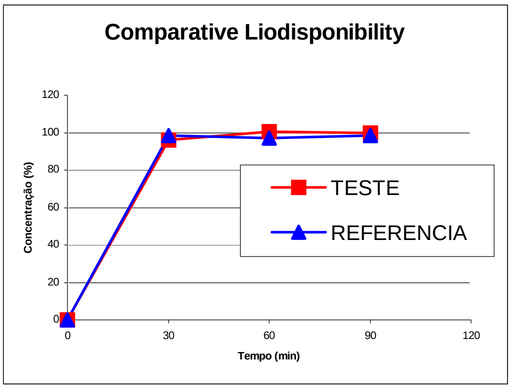

Figure 1. Comparative Liodisponibility of test and reference products.

Conclusion

This study showed the viability of the use of oyster shells, until now disposable. The tablets obtained from the oyster shell powder showed a compatible dissolution with the mark medication, commercially available. The economic viability is also incontestable and the cost of the prevention and treatment of diseases such as osteoporosis can be minimized without detriment of the quality.

Acknowledgments

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PP-04 TECNOLOGY DEVELOPMENT OF AQUEOUS SUSPENSION OF *BACILLUS SPHAERICUS* 2632 AS BIOINSETICIDE

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Introduction

Strains *Bacillus sphaericus* are very effective controlling agents of a number of insect vectors of human diseases [7]. Their toxicity is related to binary proteins (51 kDa e 42 kDa) formed during the esporulation [8], which are extremely toxic for *Culex, Aedes* and *Anopheles* larvae. The soluble toxins of *Bacillus sphaericus* exert their effects on midgut epithelia by causing disruption, separation, and ploughing of columnar epithelial cells into the gut lumen. It has been found that the disruption and swelling of midgut cause the insect to die, followed by *B. sphaericus* poisoning [7].

The use of the biolarvicide of *B. sphaericus* has shown many advantages when compared to chemical insecticides: selectivity, non-target arthropods, safety for men and environment in spite of externs factors as sunlight, rain, relativity humidity, temperature, application, vehicle and non-stardandization doses at short-term and long-term [3].

This work is concerned to the development of an aqueous suspension of *Bacillus sphaericus*, with stability at ambient temperature.

Experimental Methods

- **Materials**

Microorganism: The strain of *Bacillus sphaericus* 2362 was used in the fermentation. It belongs to Antibiotics Department of Federal University of Pernambuco and was obtained from Institute Pasteur, in France, by Aggeu Magalhães Research Center (CPqAM).
Culex quinquefasciatus larvae at L_4 stage: Obtained from Aggeu Magalhães Research Center (CPqAM).
Fermented BS1 produced by Agropecuary Research of Pernambuco Corporation (IPA).
Excipients: Sodium Carboxymethylcellulose (CMC-Na), Veegum, Sodium Propionate, Propylenoglycol, Tween 80, Deionazed Water.
Equipment: Mechanical Agitate, Bain-marie, Potentiometer DIGIMED DM 21

- **Selection of Excipients**

Using an essay of efficiency against pathogens microorganisms and ***B. sphaericus***, Sodium Propionate was selected as preservative. In sequence, one plane of qualification and quantification of excipients, 6 pilots were prepared using CMC-Na or Veegum, at different concentrations, as a suspending agent. (Table 1)

- **Preparation of Aqueous Suspension**

The fermented was centrifuged at 3,500 rpm during 30 minutes; after that, the sediment was recuperated. All components were weighted, the CMC or the Veegum were dispersed in deionized water at 50 °C, under agitation [5]. The propylenoglycol was added and homogenized. Then, the sodium of propionate, the tween 80 and finally the sediment of fermented BS1 were added, at a temperature of 30°C. Finally, the formulations were conditioned in an amber glass bottle, at room temperature. The analyses of pH, Viscosity and Biological Activity were done.

- **Determination of Biological Activity in Laboratory**

The tests were done in recipients within 100mL of distillated water and 20 *Culex quinquefasciatus* larvae. The formulations were submitted to serial dilutions until the determination of the lethal concentration media LC_{50}. Different volumes were distributed into water surface, for simulated field conditions in a laboratory. The essay was done in triplicate, with a control group, and the food was added to all of them. The results were read after 48 hours, by counting survival larvae, at 28 ± 1°C [4,6].

Results and Discussion

All formulations have shown characteristic smell and color, similar to the fermented ones. The loose sediment observed was easily redispersable, under agitation. When observed by microcopy, the aqueous suspensions have shown spores uniformly distributed and without any contaminants. The viscosity was increasing proportional to the variation of concentration of the suspensors agents. The formulations were obtained at pH values favorable to the maintenance of stability and biological activity. Among the tested formulations, the PV was selected for the biological activity assay. So, it was better attented to all specifications previously defined. (Table 2) . The biological activity result was LC_{50} of 5 ppm. This result showed that the PV is more concentrated than the fermented, then it will be more economical for government programs.

Table 1. Formulations in %

Components	P I	P II	P III	P IV	P V	P VI
Bs	*	*	*	*	*	*
CMC	0.25	0.5	0.75	---	---	---
Veegum	---	---	---	0.25	0.5	0.75
Sodium Propionate	2.0	2.0	2.0	2.0	2.0	2.0
Propylenoglycol	20	20	20	20	20	20
Tween 80	0.5	0.5	0.5	0.5	0.5	0.5
Deionizaded Water	100	100	100	100	100	100

* Sediment correspondent for 200 mL volume.

Table 2. Results of Quality Control of the Formulations

Formulations	Aspect of Sediment	Viscosity	pH
P I	Loose	40 cP	5.5
P II	Loose	95 cP	5.76
P III	Compact	120 cP	5.78
P IV	Loose	50 cP	5.62
P V	Loose	80 cP	5.87
P VI	Loose	110 cP	6.04

Conclusion

After the choice of the formulation PV as an ideal product for the larvae mosquito control, the determination of the biological activity at field will be done and, subsequently, a scale-up of the formulation.

Acknowledgements

We would like to thank those institutions that contributed for the completion of this work: LAFEPE, Antibiotics of Department (UFPE), Aggeu Magalhães Research Institute, Agropecuary Research Center of Pernambuco (IPA) and CAPES.

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PP-05 *IN VITRO* PERMEATION STUDIES OF 5-AMINOLEVULINIC ACID ESTER DERIVATIVES THROUGH HAIRLESS MICE SKINS

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Introduction

5-aminolevulinic acid (5-ALA) is a small and soluble molecule that can be converted *in situ* into the endogenous photosensitizer protoporphyrin IX (PpIX) by the heme cycle. The combination of a photosensitizer agent and oxygen is able to produce extensive tumor tissue destruction upon absorbing light of an appropriate wavelength and dose. The concept of photochemical sensitization resulting in cell death is named Photodynamic Therapy (PDT), a new cancer therapeutic modality. At present, 5-ALA seems to be the most frequently used topical agent for PDT [1]. However, hydrophilic and charge compounds, such as 5-ALA, present limited capacity to cross biological barriers. A way to overcome the limited bioavaibility of 5-ALA may be the use of penetration enhancer [2] and lipophilic 5-ALA prodrugs, like 5-ALA ester derivatives, capable to cross cellular membranes more easily than 5-ALA [3]. After entering the site of action, esters are converted enzymatically to 5-ALA, which in its turn, is converted into PpIX. Several 5-ALA ester derivatives have been synthesized with C_1-C_8 aliphatic alcohol side chains [3,4]. In this work, we determined the *in vitro* skin permeation profile of the derivatives methyl- and hexyl-esters of 5-ALA.

Methods

- **Formulations**

The formulations used in these studies were oil-in-water emulsions containing 2% 5-ALA ($C_5H_9ClNO_3$), methyl- (M-ALA, $C_5H_9ClNO_3$-CH_3) or hexyl-ester (H-ALA, $C_5H_9ClNO_3$-C_6H_{13}) (w/w). All formulations were tested in the presence of 3% EDTA (w/w).

- **Permeation studies**

In vitro permeation studies were carried out at 37°C using full-thickness of hairless mice skins and a modified Franz diffusion cell (Microette-Hanson Research). Isotonic pH 5.0 phosphate buffer was used as acceptor medium, from which samples were removed at regular intervals up to 12 hours. The donor medium was oil/water emulsions described before. The concentrations of 5-ALA and its esters in the receptor medium were determined based on Oishi *et al*. [5]: after derivatization reaction of the samples with acethylacetone and formaline, they were submitted to HPLC analysis, using a RP-18 125mm column at 40°C, methanol/water as mobile phase, 1ml/min of flux, and fluorimetric detection (excitation/emission: 378/464 nm). All procedures were performed under subdued light.

Results and Discussion

Figure 1 shows the *in vitro* permeation profiles of 5-ALA and its esters through hairless mice skins. It can be observed that H-ALA has a significant increased permeation compared to 5-ALA and M-ALA. The enhanced absorption must be due to an increase in drug diffusivity across the skin caused by the aliphatic chain C_6.

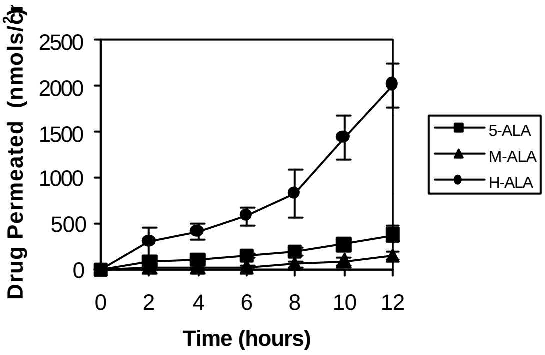

Figure 1: *In vitro* permeation profiles of 5-ALA and its esters through hairless mice skins after 12 hours: (☐) 5-ALA; ( ) M-ALA; (●) H-ALA. Averages ± SD of results obtained from 6 skin samples in each group are presented.

Table 1 shows the *J* fluxes for 5-ALA and the esters derivatives through hairless mouse skin.

Table 1.
Kinetics characteristics for *in vitro* permeation curves of 5-ALA, M-ALA and H-ALA (nmoles/cm^2) until 12 hours.

Drugs	***J* Fluxes (nmols/cm^2/h)**	**r**
5-ALA	28.419	0.9784
M-ALA	11.253	0.9285
H-ALA	155.030	0.9573

The physical-chemical properties of M-ALA are similar to 5-ALA [3], but 5-ALA flux was ~2.5 times higher than M-ALA. Probably additional charges on M-ALA molecule could be interfering on its permeation, or M-ALA could be being retained into the skin.

The major limitation of topical 5-ALA-PDT may reside in the extent of 5-ALA penetrability into the *stratum corneum.* The ability of 5-ALA to cross this membrane is of fundamental importance for it conversion into PpIX. Suitable PpIX production and accumulation in tissues could be a crucial factor for the success of PDT.

The results obtained in this work lead to consider that a promising solution for the limited skin penetration of 5-ALA, may be the use of its ester derivatives, having higher lipophilicity.

Conclusions

The H-ALA showed a significant increased permeation though full-thickness of hairless mouse skin compared to 5-ALA and M-ALA, due its higher lipophilicity caused by the aliphatic chain C_6. Altering the lipophilicity of 5-ALA molecule by esterification seems to be an interesting alternative to overcome its poor bioavaibility for topical PDT.

Acknowledgements

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PP-06 EFFECT OF MOLECULAR SIZE ON THE CATIONIC IONTOPHORETIC TRANSPORT OF A SERIES OF AMINOLEVULINIC ACID ESTER DERIVATIVES INTO AND THROUGH THE SKIN

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Introduction

Topical application of 5-aminolevulinic acid (ALA) associated with photodynamic therapy (PDT) is an experimental treatment for skin cancer currently under investigation in many laboratories. ALA is a prodrug, i.e., the photosensitizer protoporphyrin IX (PpIX) is formed *in vivo* after application and absorption of ALA [1].

The principal limiting factor which undermines the clinical efficacy of topical ALA-PDT is the poor permeation of the prodrug to the site of action [2]. There is an important research effort, therefore, directed to the enhancement of ALA delivery into the skin. Iontophoresis is a promising option, which has been shown to significantly increase ALA transport. At pH 7.4, the mechanism is primarily electroosmosis of the zwitterionic form of ALA; lowering the pH to 4 allows the cationic form of the prodrug to be transported by electromigration, but the resulting neutralization of the skin's negative charge under these conditions counteracts the expected benefit [3].

A potentially interesting strategy, by which to improve ALA iontophoretic delivery, is to use simple alkyl esters of ALA that (a) will be net positively charged at pH 7.4 (and hence more likely to be efficiently delivered by electrotransport), and (b) may be expected to be readily hydrolyzed back to the parent ALA in viable skin by non-specific esterases [4].

Goals

To determine the iontophoretic delivery of an homologous series of cationic ALA esters. The penetration of ALA and its esters into the skin were also studied.

Experimental Methods

ALA and ALA methyl ester (ALA-Me) were purchased commercially. The ethyl (Et), butyl (Bu), hexyl (Hex) and octyl (Oct) esters of ALA were synthesized with a purity >95%. The skin was dermatomed from the porcine ear (~700 µm) and stored frozen for a maximum of 7 days before use.

Iontophoretic transport of ALA, ALA-Me, ALA-Et, ALA-Bu, ALA-Hex and ALA-Oct was followed *in vitro* for 2 hours at constant current (0.5 mA/cm^2). Flow-through diffusion cells and Ag/AgCl electrodes were used [5]. The anode formulations were 15 mM ALA or ALA ester (as the HCl salt) in water to which a minimum of NaOH was added to adjust the pH to ~7. To ensure the presence of sufficient chloride in the donor compartment, the entire anodal formulation was removed and replaced after 1 hour with fresh 15 mM drug. The cathodal and receptor chambers of the diffusion cell contained physiological saline buffered with 25 mM HEPES to pH 7.4. The receptor was perfused continuously at 2 mL/h, and samples were collected automatically each 30 minutes and analyzed for transported drug by HPLC with fluorescence detection.

After 2 h of iontophoresis, the skin was removed from the diffusion cell and pinned on a piece of Parafilm™ with the *stratum corneum* (SC) faced up. The part of skin that had been in contact with the anode (0.8 cm^2) was then tape-stripped 15 times using a scotch. The tape strips were subsequently immersed in 15 ml methanol in a vial for 24 h and, finally, analyzed in the HPLC to evaluate the amount of ALA or its esters in the SC. The remaining skin was cut in small pieces and homogenised in ultra-sound for 60 seconds with MeOH. The quantity of ALA in the [epidermis + dermis] was determined, from an aliquot of the filtered homogenate, by HPLC with fluorescence detection.

Results and Discussion

Figure 1 presents the anodal iontophoretic transport of ALA and its methyl through octyl esters, in 2 hours, from a 15 mM solution of the drug, pH ~7. Iontophoresis of the positively charged ALA-Me is enormously enhanced over that of the zwitterionic ALA. As the molecular weight and the lipophilicity of the esters increased, their transport progressively decreased.

Clearly, conversion of the net uncharged ALA to the methyl ester resulted in a small, rather mobile, cation, which (there being essentially no competing positively charged species in the anode formulation) was able to transport by electromigration a significant fraction of the total charge passed between the electrodes. The transport number of ALA-Me, in fact, was greater than 0.2.

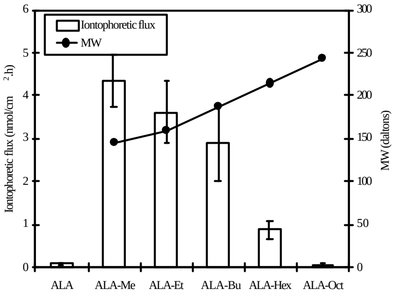

Figure 1**.** Anodal iontophoretic fluxes and molecular weights (MW) of ALA and ALA esters from aqueous solution at pH ~7 (mean ± SD; n = 8).

Figure 2 demonstrates that iontophoresis of cationic ALA-Me and ALA-Hex not only increase the flux across the skin but also the amount of prodrug delivered into the skin (SC and [epidermis + dermis]) when compared with the iontophoresis of the zwitterionic ALA.

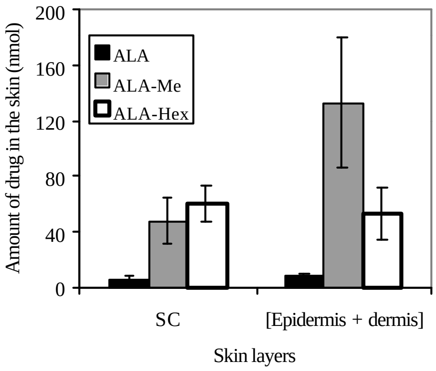

Figure 2. Amount of ALA, ALA-Me and ALA-Hex into the skin (SC and [epidermis + dermis]) after 2 hours of iontophoresis (mean ± SD; n = 5).

Conclusions

In summary, the significant enhancement of ALA delivery into and through the skin is possible by iontophoresis of the drug's cationic esters. Iontophoresis enhances ALA-Me and ALA-Hex transport across and, more importantly, into the [epidermis + dermis] than the parent drug, ALA.

Acknowledgments

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PP-07 *IN VITRO* PERCUTANEOUS ABSORPTION OF PAROMOMYCIN, AN ANTI-LEISHMANIAL AMINOGLYCOSIDE, FROM HYDROPHILIC FORMULATIONS

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Introduction

Paromomycin (PA), an aminoglycoside antibiotic have been tested in an ointment (PA15% and metylbenzethonium chloride 12%) as an alternative treatment against cutaneous leishmaniasis [1]. Although the influence of the vehicle on the topical delivery is important for optimisation of topical formulations containing PA, it has been little investigated experimentally. It has been showed that the in vitro percutaneous absorption of PA from O/W cream was higher than that from ointment, in presence or absence of *stratum corneum* (SC) [2]. However, cream containing a concentration relevant clinically of PA (10% or plus) was not stable. Thus, we developed a hydrophilic gel.

The aim of this study was to evaluate the influence of the vehicle (O/W cream × gel) and viscosity on the in vitro topical delivery of PA.

Experimental methods

- **Materials**

Paromomycin sulfate (PA), potency of 718 mg/g, (Pharmacia & Upjohn, Italy), paraffin oil (Favab, Brazil), cetyl alcohol (CA), glyceryl stearate (Henkel, Germany), PEG-40 hydrogenated castor oil [Cremophor RH 40 (CR), BASF, Germany] and hydroxyethyl cellulose (Natrosol, Aqualon, EUA). All other chemicals used for analysis were analytica reagent grade.

- **Preparation of formulations**

The base of the O/W cream was prepared according to the following formula: oily phase - 16% of paraffin oil, 7% of cetyl alcohol, 2% of glyceryl stearate; aqueous phase - 3% of PEG-40 hydrogenated castor oil (CR) and 72% of distilled water, by weight. The two phases of the O/W cream were prepared separately. Both phases were heated to 75^{o}C and the aqueous phase was added to the oily phase. After cooling at room temperature, PA was incorporated into the O/W cream.

The gel was prepared by hydrating Natrosol (2%) in distilled water heated at 70°C alone or with 2% of CR. After cooling at room temperature, PA was incorporated.

- ***In vitro* percutaneous absorption**

In vitro percutaneous absorption of PA was determined with an open-cap, ground-glass surface, modified Franz diffusion cells (membrane surface area of 1.77 cm2 and volume of the receptor fluid of 6.7 cm^3). Dorsal skin was excised from hairless mice. Animals were sacrificed by cervical rupture and the dorsal skin was used immediately after removing the subcutaneous fat.

These experiments were made in conditions relevant clinically (absence of SC). The SC was removed by stripping 10 times with a cellophane tape (SCOTH Magic no 810, 3M). The skin was kept horizontally dividing the cell into two compartments: the donor and the receptor. The receptor compartment was filled with 0.05M phosphate buffered saline (pH 7.4) containing 0.01% of HgCl2 as preservative. The receptor phase, maintained at 37oC, was continuously stirred with a small magnetic bar to ensure homogeneity.

The preparations (100 mg), containing 1% w/w of PA, were applied on the skin. Serial sampling was then performed at specific times by totally removing the receptor fluid and refilling with fresh solution. The concentration of PA in receptor fluid was determined by microbiologic assay.

- **Microbiologic assay of the PA**

The concentration of PA was determined by an agar-diffusion inhibition assay of the growth of *Bacillus subtilis* (ATCC 6633), as described previously [3]. Briefly, after overnight incubation (by 18 h) of the plates at 35^{o}C, the zone of inhibition was measured for each sample. A straight-line relationship was obtained between drug concentration (µg/ml) log and zone of inhibition (mm). The concentration of PA was determined from a standard curve ($y = 17.59 + 8.258x$; $r^2 = 0.985$).

Results and discussion

The Figure 1 shows the influence of the concentration (2 or 7 %) of cetyl alcohol (CA) on the percutaneous absorption of PA from O/W cream.

After 8 hours, the absorption of PA (% applied dose) from O/W cream containing 2% CA (71.7 ± 3.3) was significantly higher than that observed from cream containing 7% CA (54.5 ± 3.3).

It well known that the viscosity has little influence on topical delivery across intact skin. However, ours results have shown that, in absence of SC, the lowest the viscosity the highest is the PA absorption and lowest is the stability. Thus, the O/W cream more viscous (7% CA) was chosen for more studies.

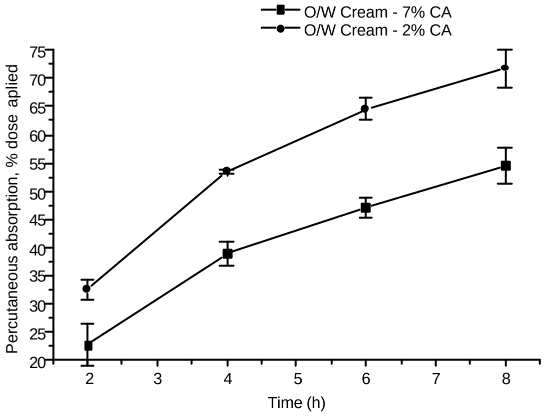

Figure 1: Percutaneous absorption of PA from O/W cream containing 2 or 7 % of CA. The formulations containing 1% w/w of PA were applied over biopsies of hairless mouse skin, in absence of SC (10 strippings), mounted in Franz cells. Each point represent average (n = 3) ± SD.

The PA absorption from hydrophilic gel was higher than that observed from O/W cream (Figure 2). This difference was significant (ANOVA; $p<0.05$). The presence of a hydrophilic surfactant (CR) increases the PA absorption from gel, but this difference was not significant.

Although the difference between cream and gel has been significant, it was lower than that observed when comparing the cream and the ointment. The difference between this two formulations was 2.5 and 10 times in the partial and total absence of SC, respectively [2].

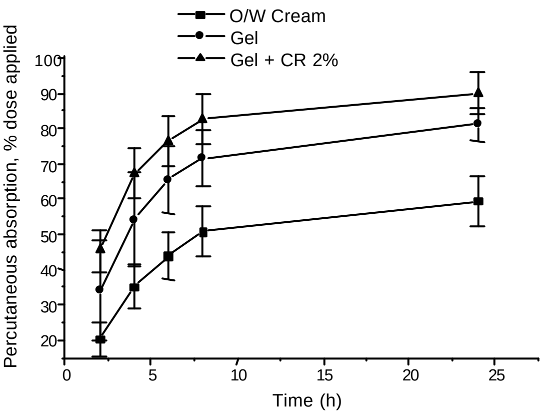

Figure 2: Percutaneous absorption of PA from O/W cream and gel. The formulations containing 1% w/w of PA were applied over biopsies of hairless mouse skin, in absence of SC (10 strippings), mounted in Franz cells. Each point represents average (n = 3 to 6) ± SD.

Conclusion

The PA absorption across stripped skin was influenced by the viscosity of the O/W cream. The lowest the viscosity the highest is the PA absorption. The absorption from hydrophilic gel was higher than that from O/W cream. The presence of a surfactant in the gel has little influence on the PA absorption.

Acknowledgments

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PP-08 THE INFLUENCE OF THE VEHICLE ON THE *IN VITRO* PERCUTANEOUS ABSORPTION OF NIMESULIDE

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Introduction

Nimesulide (NI), an acidic non-steroid anti-inflammatory drug (NSAID), acts mainly through the selective inhibition of cyclooxygenase-2 (COX-2). NI shows high anti-inflammatory, antipyretic and analgesic activity in addition to low toxicity [1]. The anti-inflammatory has been developed as topical formulations due to side effects associated with oral or parenteral administration [2].

Gupta *et al*. [3] demonstrated that a NI topical gel showed similar anti-inflammatory activity when using carrageenan paw edema model in comparison to diclofenac gel (DF). However, NI was more effective than DF when using the formalin-paw edema model. Sengupta *et al.* [4] evaluated the antinociception induced by NI gel on the mice skin. The findings of this study suggest that NI gel provide significant analgesic activity when applied topically. A study in human [5] showed superior analgesic activity of NI gel compared to piroxicam, DF and placebo. However, the influence of the vehicle on topical delivery of NI has not been investigated.

The aim of this study was to evaluate the influence of various parameters on the *in vitro* percutaneous absorption of NI: the influence of the stratum corneum (SC), the composition of receptor liquid medium and the effect of skin penetration enhancers.

Experimental methods

- **Materials**

Nimesulide (Heartwell S.A.), Polyethylene glycol 40 hydrogenated castor oil/ CR (Cremophor RH 40), propylene glycol/ PG (BASF, Germany), ethanol/ ET and diethyleneglycol monoethyl ether/ TCL (Transcutol CG – Gattefossé). All other chemicals used were of analytical or HPLC grade.

- **Saturated solution of NI**

Saturated solution of NI was prepared by suspending an excess of drug in 10 mL of each solvent separately: PG, TCL and ET. The suspensions were stirred under magnetic agitation for 24 h at 25°C. The sample was centrifuged and taken an aliquot of supernatant.

- ***In vitro* percutaneous absorption**

In vitro percutaneous absorption of NI was determined with an open-cap, ground-glass surface, modified Franz diffusion cell (membrane surface area of 1.77 cm^2 and volume of the receptor liquid medium of 6.7 cm^3). Dorsal skin was excised from hairless mice. Animals were sacrificed by cervical rupture and the dorsal skin was immediately used after removing the subcutaneous fat. These experiments were made in the presence or absence of the SC. The SC was removed totally by stripping 15 times, with a cellophane tape (SCOTH Magic n° 810, 3M). The skin was kept horizontally dividing the cell into two compartments: the donor and the receptor. The receptor compartment was filled with 0.05M phosphate buffered saline (pH 7.4) containing 0.01% of $HgCl_2$ as preservative and 2% of CR, to improve the solubility of NI in aqueous solution. The receptor phase, maintained at 37°C, was continuously stirred with a small magnetic bar to ensure homogeneity. The skin, mounted in the cell, was allowed to equilibrate with the environment for 1 hour. After this period, the receptor liquid medium was totally removed and this compartment was filled with fresh solution.

In order to simulate use conditions, the upper chamber (donor compartment) was left open, so that, volatile components of the preparation could escape. A 500 µL aliquot of saturated solution of NI was applied on the skin using a calibrated Stepper 411 pippetor. Serial sampling was then performed at specific times by totally removing the receptor fluid and refilling with fresh solution. The concentrations of NI in the receptor liquid medium were determined by high performance liquid chromatography (HPLC).

- **Determination of NI by HPLC**

The concentrations of NI in the receptor liquid medium were determined by HPLC in the following conditions: isocratic system with a UV detector (Waters) at 300 nm, Supelcosil C18 reversed-phase column (25 cm × 4.6 mm, 5 µm particle size), automatic sampler, 20 µL injection volume and flow-rate of 1 mL/min. The mobile phase was 0.05

M phosphate buffer pH 5/acetonitrile (40:60 v/v). In these conditions the drug eluted at about 6.0 min. The limit of detection and quantification were 0.01 and 0.05μg/mL, respectively.

Results and discussion

The influence of the composition of receptor liquid medium was investigated (Table 1). The presence of CR in the receptor compartments increased significantly the NI topical delivery (ANOVA; $p<0.05$). Probably, it was due to the increase of the NI solubility in the receptor liquid medium. The aqueous solubility of NI was of 10 μg/mL, whereas its solubility in aqueous CR at 2% was of 130 μg/mL.

Table 1. Cumulative amounts of NI (μg)
(Mean ± standard deviation)

Time (h)	Absence CR	Presence 2% CR
4	ND	0.3 ± 0.1
6	ND	0.8 ± 0.1
8	ND	1.3 ± 0.1
24	2.5 ± 0.1	9.2 ± 1.9

Donor phase: NI saturated solution in PG
Number of hairless mice/ group (n) = 3
ND = not detected

In the absence of SC, the amount of penetrated NI was about 15-fold higher than that observed in the presence of SC (Table 2). This result shows that the SC was the principal barrier to NI penetration through the skin.

Table 2. Cumulative absorption of NI (μg)
(Mean ± standard deviation)

Time (h)	Presence SC	Absence SC
4	ND	4.7 ± 2.9
6	ND	6.7 ± 3.5
8	ND	8.9 ± 3.8
24	1.3 ± 0.5	19.6 ± 4.1

Donor phase: NI saturated solution in PG
Number of hairless mice/ group (n) = 4
ND = not detected

The relative impermeability of the SC provides the main resistance to percutaneous absorption of the most drugs. Attempts to improve permeability have been made using skin penetration enhancers to reversibility reduce this barrier resistance. The results in Table 3 demonstrate the effect of various enhancers (PG, TCL and ET) on the percutaneous absorption of NI. The NI absorption from ET was higher than that observed for TCL and PG. These differences were significant (ANOVA; $p<0.05$). The absorption from TCL was higher than that from PG, however this difference was not significant.

Table 3. Cumulative amounts of NI (μg)
(Mean ± standard deviation)

Time (h)	PG	ET	TCL
4	ND	1.7 ± 0.1	0.4 ± 0.1
6	ND	3.5 ± 0.2	0.8 ± 0.1
8	0.3 ± 0.1	4.1 ± 0.6	1.3 ± 0.1
24	2.1 ± 0.2	4.6 ± 0.8	2.8 ± 0.1

Donor phase: NI saturated solution in PG, ET and TCL
Number of hairless mice/ group (n) = 3
ND = not detected

Conclusion

The presence of a surfactant (CR) in the receptor liquid medium improved the solubility and permeation of NI across intact skin. The NI absorption in the absence of SC was higher than in its presence. This result demonstrated that the SC was the main barrier for NI permeation across intact skin. The enhancing effect of PG, ET and TCL on the permeability of NI, showed that the maximum activity was obtained with drug in ET, followed by TCL and PG.

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PP-09 RINGING GEL: STUDY OF FORMATION AND CHARACTERISATION THROUGH BOTH POLARISING MICROSCOPY AND ELECTRIC CONDUCTIVITY

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Introduction

For skin care and topical treatment of dermatological diseases, there is a great number of vehicles to be chosen, such as solid, semisolid and liquid compounds, which are available both clinician and patients. Among the semisolid groups, gel usage is widespread both in the cosmetic field and pharmaceutics field [1,2].

Ringing gels can be described as semisolid systems formed by water, oil and one or more emulsifying agents. They present a gelatinous consistency, are opaque or transparent, clear and homogeneous, optically anisotropic and thermodynamically stable. The ressonant feature takes place when the recipient which contains the gel is moved, provoking vibrations. According to the literature, many terms are used in order to name this kind of gel. Among the most common designations, we could cite: microemulsion gel, transparent emulsion gel, resonant gel, viscose isotropic phase and transparent oil-water gel [1,3].

Our aim is to study the formation of ringing gels from ternary systems constituted by oleth-3-phosphate (surfactant), canola oil and isopropyl myristate (lipophilic phase) and distilled water as well as to define them by using both polarising microscopy and electrical conductivity.

Experimental methods

- **Materials**

The following materials were used: oleth-3-phosphate (equimolar mixture of mono- and di-ester of phosphoric acid and oleilic alcohol) as anionic surfactant marketed by Hoechst in Brazil, as lipophilic compounds, canola oil (Olvebra) and isopropyl myristate (Aquatec) and as hydrophilic compounds, newly distilled water.

- **Phase Diagram**

The phase diagrams were obtained from the method described by LO *et al.* [4] with some modifications. The diagrams were defined according to their compound concentration of 10 by 10%.

- **Sample Preparation**

Enough samples were prepared according to the chosen concentration of oil-surfactant-water. The oil-surfactant mixture was heated in a double-boiler at 70 ± 5°C, in which distilled water was added at the same temperature. After shaking manually during 15 minutes, it was kept at ambient temperature until equilibrium.

- **Polarising Microscopy**

The photomicrographs of crystalline mesophases concerning ringing gels were obtained through experiments by using an OLYMPUS model BX50 polarising microscope. The ringing gel sample was previously heated at 45°C for approximately 30 minutes (after referred temperature) in a PHYSITEMP model TS-4 CONTROLLER heating platform fixed to a double-boiler with a FANEN model 100 water circulator before being interposed between the slide and the thin slide.

- **Electric Conductivity Measures**

Specific conductivity measures for ringing gels were taken by means of ANALION model C-701 conductivimeter and one microcell presenting a constant value of 1 ± 10%, depth of 30 mm and diameter of 12/120 with two platinum electrodes. The resonant gels from each diagram were submitted to electric conductivity analysis at 25°C, and such temperatures were automatically adjusted by equipment. The constant measure of specific conductivity from ringing gels under thermal treatment was taken in the same conductivimeter, and the samples were heated and then cooled in an ultra-thermostatic bath by using NOVA TECNICA model NT 281 device at 5°C to 45°C.

Results and discussion

In the phase diagrams of ternary systems formed by oleth-3-phosphate, canola oil (Figure 1) and isopropyl myristate (oiled phase) and water, different pharmaceutic/cosmetic forms were identified.

Necessary concentrations for ringing gel achievement were >25% of oleth-3-phosphate, <20% canola oil and <80% of distilled water (system in Figure1) and 40 - 70% of oleth-3-phosphate, <20% isopropyl myristate and 20-40% of distilled water.

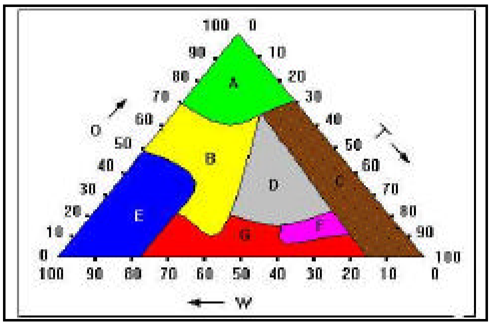

Figure 1: Ternary system formed by oleth-3-phosphate, canola oil and water. A oiled dispersion; B: instable dispersion; C: Oiled isotropic ; : Non-rigid gel; F: Rigid gel; G: Ringing gel; E: Emulsion.

After polarising microscopy analysis of the system formed by oleth-3-phosphate, canola oil and water, we have identified, in samples 43 (surfactant 55%; oil 5%; water 40%) and 45 (surfactant 45%; oil 5%; water 50%), the presence of either hexagonal liquid crystalline phase (Figure 2).

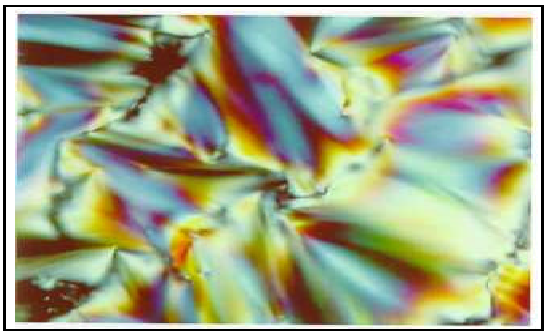

Figure 2: Photomicrograph showing hexagonal crystalline phase formed by oleth-3-phosphate, canola oil and water.

In the ternary system formed by oleth-3-phosphate, isopropyl myristate and water, where samples 25 (surfactant 40%; oil 20%; water 40%), 30 (surfactant 70%; oil 10%; water 20%), 31 (surfactant 60%; oil 10%; water 30%) and 32 (surfactant 50%; oil 10%; water 40%) was examined by using polarising microscopy, we have found the formation of either hexagonal liquid crystalline phases.

The results from electric conductivity measures for ringing gels formulated from phase diagrams of systems constituted by oleth-3-phosphate (surfactant); canola oil, isopropyl myristate (oiled phase) and distilled water are briefly shown in Table I.

Table I: Electric conductivity of ringing gels.

OIL	OLETH-3-PHOSPHATE	
	Sample	Conductivity mS/cm^{-1}
Canola oil	43	3.25
	45	4.22
Isopropyl miristate	25	0.30
	30	0.56
	31	0.13
	32	0.93

In Table I, we can observe that conductivity values of ringing gels are about 1 mS/cm^{-1}. According to values reported by Nurnberg, 1980 [2], this indicates that such gels are formed by water during constant external phase. From the point of view of usage, resonant gels could be characterised for good removability and adhesiveness to hydrophilic support like a mucosa. Under thermal treatment, ringing gels which were tested present similar behaviour regarding their specific conductivity, that is, increase in temperature raises conductivity instantaneously. A decrease in temperature causes the contrary.

Conclusion

This study shows the obtention or ringing gel from phase diagram composed by anionic surfactant – canola oil or isopropyl myristate – water and its characterisation by polarising microscopy and electric conductivity. It was found that the presence of hexagonal crystalline phase depends on equilibrium among phase diagram components. Under thermal treatment, behaviour regarding their specific conductivity, that is, increase of temperature raises conductivity instantaneously.

Acknowledgements

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PP-10 TERNARY PHASE DIAGRAM OF GLYCERYL MONOOLEATE/WATER SYSTEMS IN THE PRESENCE OF SALYCILIC ACID AND DRUG RELEASE

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Introduction

Lipids materials are emerging as new excipients presenting several interesting features for pharmaceutical applications. Glyceryl monooleate (GMO) is a polar lipid that forms gels with a lyotropic liquid crystalline phase structure in the presence of water, depending on the water content, temperature and the presence of solutes. GMO/water systems are non-toxic, biocompatible and can incorporate both hydrophilic and lipophilic drugs, being suggested as drug delivery systems for drugs with different characteristics. In addition, they had shown the ability to sustain drug delivery [1]. The presence of a drug, however, can influence the liquid crystalline phase presented by the systems and, therefore, alter the drug release profile of the system.

Objective

The aim of this work was to characterize the liquid crystalline phases from GMO/water systems in the presence of salicylic acid (SA) as well as to study in vitro drug delivery from these systems evaluating the effect of drug load and drug solubility in the systems.

Experimental Methods

The following reagents were used as received: a commercial grade of GMO, Myverol 18-99 distilled monoglycerides (Eastman Chemical Company) and SA (Sigma Chemical Company).
GMO/water systems were prepared at several water content varying in the range of 5 to 40% (w/w) by melting GMO at 42ºC, mixing SA in the molten GMO, followed by the addition of the required amount of water at the same temperature. The systems were allowed to equilibrate at room temperature for 7 days and polarizing light microscopy at 37ºC identified the liquid crystalline phases.
An indication of SA solubility in the systems was achieved by the presence of drug crystals in the systems when observed at the microscope.
For *in vitro* drug release studies cubic phase GMO/water systems were prepared containing several SA loading in the range of 1 to 16% (w/w) which comprises systems containing both drug dissolved and not.
The *in vitro* drug release studies were carried out based on the rotating basket method (USP) using isotonic phosphate buffer pH 7.4 as dissolution media at 37°C and 100rpm agitation. The amount of SA released was assessed spectrophotometrically at 296nm.

Results and Discussion

Figure 1 presents the ternary phase diagram obtained in this study. It was observed the presence of an isotropic liquid and the formation of lamellar phase and cubic phase, respectively, with increasing water content. Lamellar phase was found at low water content, until 10.0% w/w of water. In the systems with water content of 15.0% w/w it was observed both lamellar and cubic phase, and greater water contents systems presented cubic phase structure.

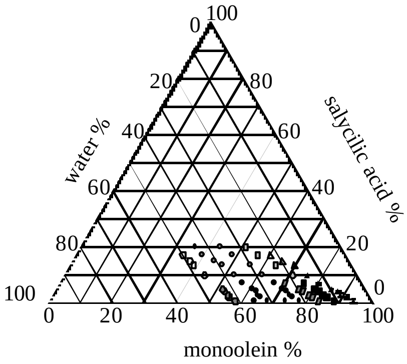

Figure 1 – Ternary phase diagram of GMO/water/SA.

Legend: (▲) isotropic liquid;
(△) isotropic liquid + crystals;
(■) lamellar phase;
(⬡) lamellar phase + crystals;
(▰) cubic phase + lamellar phase;
(●) cubic phase;
(◉) cubic phase + crystals);
(⬢) cubic phase + water
(⬡) cubic phase + water + crystals.

GMO reaches its water content equilibrium water content at 40.0% w/w, presenting cubic phase structure, which is stable in excess of water.

These results are in agreement with the mesophases found in binary phase diagrams reported for GMO/water systems [1,2], indicating that the presence of SA in the range of loading used in this study had no effect on the liquid crystalline phases presented by the systems.
Figure 2 presents the *in vitro* SA release profiles from cubic phase GMO/systems.

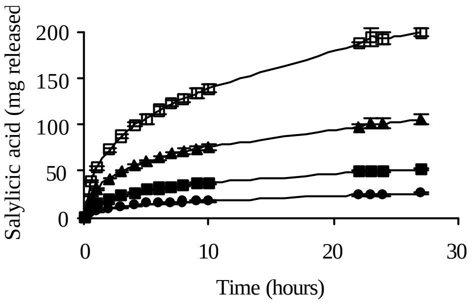

Figure 2 – Plots of the cumulative amount of SA released from cubic phase GMO/water systems containing: (●) 2.0 % (w/w) of SA; (■) 4.0% (w/w) of SA, (π) 8.0% (w/w) of SA and (⊠) 16.0% (w/w) of SA.

The drug release was similar for all the systems studied, including those systems with unsolubilized drug, indicating that the release was independent of the drug load. The percentage of SA released was similar for all the systems studied and it was released around 92.0% of the SA present in the samples after 27 hours. The drug release was found to be linear with square root of time, indicative of diffusion controlled release in all systems studied.

Conclusions

-the presence of SA at the drug loads studied did not modify mesophases presented by GMO/water systems
-cubic phase GMO/water system provide a diffusion controlled release of SA, which was independent of drug load and drug solubility in the system.

Supported by

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PP-11 RHEOLOGICAL EVALUATION OF POLOXAMER 407 GELS FOR UNDERSTANDING OF THE IN SITU THERMAL GELIFICATION

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Introduction

Poloxamer 407 is a copolymer that have many pharmaceutical uses. It has a molecular weight of 12.500 Daltons and a PEO/PPO ratio of 2:1. Due to the reverse thermal gelification and low toxicity, the use of the polymer has been recently suggested in the pharmaceutical field for controlled drug release. In aqueous solution, with increasing temperature, Poloxamer 407 aggregate in micelles to minimize the free energy of solution. In the low temperature, Poloxamer 407 existed as unimer in solution. Upon warming, in an equilibrium between unimers and micelles is stablished and aggregates are formed at higher temperatures [1].

Lidocaine is widely used for treatment of acute and chronic pain, but their usefulness is limited by the short duration of action. A long-action sigle-dose injection would be of clinical importance and methods that extend the action of local anesthetics are important. The evaluation of thermo reversible gels for sustained release of lidocaine is therefore of interest [3, 4].

In the present work a rheological evaluation of the gels was made with differences concentrations in order to understanding of the thermal gelification *in situ*.

Experimental methods

- **Materials**

Poloxamer 407 (Pluronic F-127®) was obtained from BASF. Lidocaine was purchased from Sigma Chemical.

- **Preparation of gel solution**

The gel was made on a weight basis using the cold method. An appropriate amount of Poloxamer 407 to 20, 25 and 30% w/w was slowly added to cold water (5°C), while maintaining constant stirring. The beaker was left in the refrigerator util a clear solution was formed (6-12 hr). Lidocaine HCl (2% w/w) was dissolved in the cold solution. Poloxamer 407 gels with Lidocaine · HCl (2% w/w) was autoclaved (120°C, 30 min) and stored in the refrigerator.

- **Studies of rheological properties**

The rheological properties of the gels were studied using a Strain Control Rheometer. The rheometer is of the couette type. The cylinder is surrounded by a oven which is connected to a cylinder of liquid nitrogen. Cold polymer solutions were transferred to the cylinder (10mL). In the temperature-sweep and strain-sweep measurements, the instrument was used in the oscillatory mode.

The strain amplitude was varied in the range 0.1 to 100. It was ensured that the temperature-sweep, frequency-sweep and strain-sweep measurement were obtained in the linear viscoelastic region. Strain sweep measurements were made for some sample to determination of the maximum strain amplitude for the gel because above a certain strain amplitude the three-dimensional network of the gel is disrupted. The elasticity modulus (G') and viscosity modulus (G") were determined for frequencies between 0.1 until 100rad.s^{-1}. Viscosity measurements were made at 37°C, similar body temperature, for shear rate between 0.1 until 400 s^{-1}.

The sol-gel transition temperature of Poloxamer 407 gels was determined from oscillating measurements at 1.0rad. s^{-1} and the temperature rate was increased 2°C/min. The gelification temperature was defined as the point where the elasticity modulus was half way between G' for the solution and G' for the gel [2].

Results and Discussion

The sol-gel transition temperature for the concentration of Poloxamer solutions of 20, 25 and 30 % (w/w) with lidocaine 2% (w/w) are shown in Figure 1. The elasticity modulus, G', is low for a solution and increases drastically with temperature as a result of gel formation process. In this case elasticity modulus, G,' become independent of increase in temperature. The transition temperature decrease with increasing of concentration (Table 1).

Table 1. Sol-gel transition temperature for the Poloxamer 407 gels.

Sample	Sol-gel transition temperature (^{o}C)
Gel at 20%	17.4
Gel at 25%	12.5
Gel at 30%	9.5

The viscosities measurements are showed in Table 2. All Poloxamer gels are pseudoplastics and have a sol-gel transition temperature below 37^{o}C (Table 1). The viscosity of the gels are decreases with increasing shear rate, γ (s^{-1}), and they become fluids and this facilitate the flowage of the formulating.

Table 2. Viscosity (Pas) for Poloxamer 407 gels at 37 ^{o}C at different shear rates.

Sample	Shear rate (s^{-1})				
	0.1	1.0	10	100	400
Gel at 20%	2549	256	23	2.8	0.96
Gel at 25%	4037	418	33	4.7	1.52
Gel at 30%	4961	513	46	7.0	2.28

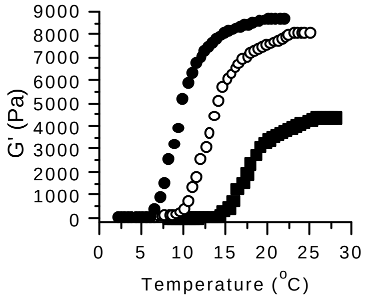

Fig. 1. Graphs of the sol-gel transition temperature: effect of the concentration. Concentration of the gels: (■) 20% , (○) 25% and (●) 30%.

The rheological properties of Poloxamer gels at 37^{o}C at different concentration are shown in Fig. 2 All formulations presented gel this temperature. Increasing the concentration increased the elasticity modulus (G') and the viscosity modulus (G") and there is a small concentration dependence. In this conditions Poloxamer 407 gels have viscoelastics properties. An increasing in the concentration of polymer result in gels more viscous (Table 2).

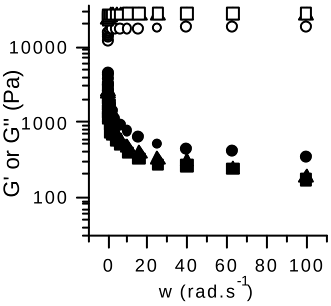

Figure 2. Elasticity and viscosity modulus at 37^{o}C as a function angular frequency. Elasticity modulus, G', unfilled symbols and viscosity modulus, G", filled symbols. Concentration of the gels: (○) 20%, (△) 25% and (□) 30%.

Conclusion

The results obtained in this work showed that Poloxamer 407 gels at 20, 25 and 30% presented different rheologic properties. The gelification temperature decrease with the increasing of concentration, which is an important characteristic for modulation of the rate of in situ gelification and consequently the lidocaine release profile from this systems.

Acknowledgements

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Controlled release gel of ibuprofen and lidocaine in epidural use analgesia and systemic absorption in pig. 13. 482-487. (1998).

PP-12 POLARIZING LIGHT MICROSCOPIC STUDIES FROM LIPID/WATER SYSTEMS

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Introduction

Polar lipids in a hydrated environment take on several morphological forms at the molecular level, depending on the degree of hydration and temperature. This multiplicity of morphologies is related to their amphipatic nature because they usually have two long hydrocarbon chains as well as a short polar group attached to the glycerol moiety; the result is that the polar group tend to interact with the solution water, whereas the hydrocarbon chains avoid the water environment. Depending on the water available, the temperature and the properties of the lipid in question, the lipids may thus form a variety of structures [1].

Liquid crystals are described as an intermediate state in the thermal transformation from the solid to the liquid state. This intermediate phase exhibits anisotropy and properties characteristic of solids and liquids. When viewed between crosses Nicol prisms of a polarizing microscope, intense color bands are seen. The liquid crystalline phase is thermodynamically stable and represents a state of incomplete melting [2].

Phospholipids are examples of susbtances that form liquid crystalline phases upon contact with water. Dioleylphosphatidylglycerol (DOPG), particularly, has this property. Glyceryl monooleate (GMO) are also a polar lipid, which exhibit a rich mesomorphism in water systems.

Various micellar and primarily anisotropic liquid crystalline phases have been evaluated for pharmaceutical applications. Among liquid crystalline phase, lamellar phase have been used in the stabilization of emulsion or cream, formation of liposomes, sustained drug delivery and the solubilization of low solubility drugs. The cubic phase has been described as a sustained-release carrier for both conventional and peptide drugs.

Among the various physicochemical techniques used to identify the mesomorphic phases, the most widely used is polarizing light microscopy, that associated with a hotstage system provide the characterization of these phases in different temperatures.

Objective

The aim of this work was to characterize phases from DOPG/Water and GMO/Water systems by polarizing light microscopy.

Experimental Methods

DOPG was hydrated with water and submitted to the microscopic analysis. The temperature of the samples was control by hotstage installed to the microscope. Photomicrographs of crystal phase were obtained from different temperatures and hydration degrees.

GMO/water systems with known amounts of water varying in the range of 5 to 40% (w/w) were prepared and observed at the polarizing light microscopy after equilibration at room temperature for 7 days. The samples were heated at 1°C/min over the temperature range of 25 to 85°C while being constantly monitored for a change in the liquid crystalline phase and a binary phase diagram was constructed.

Results and Discussion

In crystalline state, the amphiphilic molecules are oriented with the hydrophilic polar groups adjacent to each other and the lipophilic hydrocarbon chains are parallel. In contact with water and increase of temperature, the lipophilic chains are transformed into a disordered, liquid state and the water penetrates between the polar hydrophilic layers to form a lyotropic liquid crystalline structure; so various different type of aggregates structures are formed in systems consisting of a amphiphilic agent [3].

Lamellar phase consist of planar lipid bilayers stacked in one-dimensional lattice separated by layers of water and it is formed when layers of water interpenetrate polar heads groups. Cubic phase structure are transparent gels, optically isotropic and very stiff and consist of two congruent network of water channel surrounded by curved lipids bilayers extending in three dimensions. Hexagonal phase consist of water cylinders arranged in a two-dimensional lattice separated by lipid bilayers [6].

Photomicrographs of DOPG/water and GMO/ water systems are showed in Fig. 1 and 2, respectively.

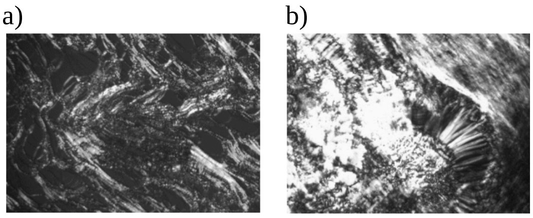

Fig.1: Photomicrographs showing the liquid crystal phases of lipid/water systems. a) Lamellar phase found on DOPG/water systems with water content of 27%w/w at 25°C after 24h equilibration. b) Hexagonal phase found on DOPG/water systems with water content of 25% w/w at 27°C.

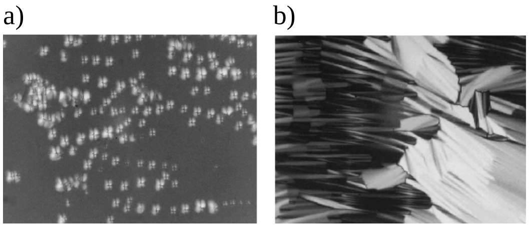

Fig.2: Photomicrographs showing the liquid crystal phases of lipid/water systems. a) Lamellar phase found on GMO/water systems with water content of 10%w/w at 25°C. b) Hexagonal phase found on GMO/water systems with water content of 25% w/w at 25°C.

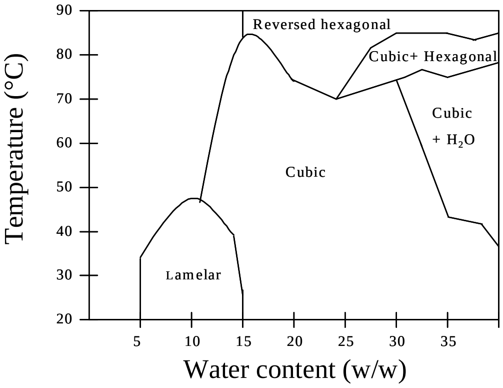

Fig.3: Binary phase diagram of GMO/water system.

Depending on the temperature and the hydration degrees studied, it was observed lamellar and hexagonal phase in DOPG/water systems. Lamellar and hexagonal phases were found at low water content and below DOPG transition temperature (37-40°C). Increasing the water content and the temperature only lamellar phase was obtained.

GMO/water systems presented lamellar, cubic, L2 and hexagonal phases. Lamellar phases were found at low water content at room temperature. On heating, these samples become isotropic and fluid, indicative of L2 structure. An increase in water content procedures gels with cubic phase. GMO/water systems reach its maximum hydration at water content of 40% (w/w) presenting cubic phase, which is stable in the presence of excess of water. At temperatures greater than 75°C, samples become anisotropic with characteristic of reversed hexagonal phase at the microscope. The binary phase diagram is presented in Fig. 3.

In addition, studies have showed that the DOPG are used as cutaneous penetration enhancers of drugs [4,5].

In conclusion, the DOPG and GMO/water systems showed opposite behaviours. Lamellar phase from GMO/water system was obtained at low content of water, while DOPG/water system showed lamellar phase at high content of water. Hexagonal phase from GMO/water system was obtained at high temperature, while DOPG/water was obtained at low temperature.

Conclusion

The results obtained suggest DOPG/water and GMO/water systems can be explored as a drug delivery system. However, release studies should be further carried out with hydrophilic and lipophilic drugs.

Supported by FAPESP

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PP-13 PHOTODECOMPOSITION OF AN ANTISEPTIC AND RADIOPROTECTOR DRUG QUINIFURYL: EFFECT OF OXYGEN AND SURFACTANT PRESENCE

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Introduction

A derivative of nitrofuran, Quinifuryl (X, Figure 1), shows high antiseptic, radioprotective and anticarcinogenic activity in model experiments with cell cultures, while its pharmacological efficacy in an organism is very low [1]. This should be explained by its fast decomposition due to metabolism in the normal tissues, especially in liver tissues. We believe that the problem should be resolved using the drug delivery technique. However, the X interaction with drug delivery systems has not yet been studied. On the other hand, X posses intense absorption in the visible spectral region (λ_{max} =396 nm). Some toxic effects of X such as allergic reactions and hemolytic anemia are attributed to its photoreactions [2]. Due to light absorption X decomposes, the processes including the X triplet state formation [3,4]. Molecular oxygen inhibits the X photodecomposition. The presence of drug delivery systems should affect this process stimulating or inhibiting the X photodecomposition and thus changing its pharmacological activity.

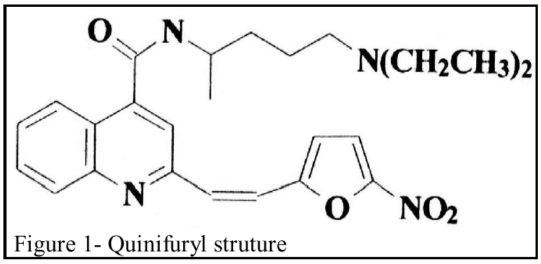


Figure 1- Quinifuryl struture

In this work we report on the study of effects of the sodium dodecylsulfate (SDS) on X photodecomposition in aqueous solutions at physiological pH in the presence and absence of molecular oxygen.

Materials and Methods

Fresh aqueous solution of X in concentration 20µM was prepared in Milli-Q water at pH 6.8-7.0. 2.0 mL of X solution in a quartz fluorescence cuvette was purged by ultra pure N_2 during 5 minutes to exclude O_2. Anionic SDS (Sigma) stock solution was added to the X solution creating the SDS concentrations in the range from 0 to 30mM.

The X photodecomposition was induced by illumination of solutions by continuum light in the region from 350 to 450 nm. The photodecomposition rate constant (k_d) was determined spectrophotometrically monitoring the decrease of the X absorbance at 396 nm. The X lifetime of the X triplet state (τ_T) characteristics were studied using flash-photolysis "LP 900 Edinburg Analytical" technique.

Results and Discussion

Figure 2 shows the effect of oxygen on τ_T. The extraction of O_2 in homogenous aqueous solution of quinifuryl increases the lifetime of its triplet state τ_T (Figure 2) and induces a dramatic increase of k_d (Figure 3).

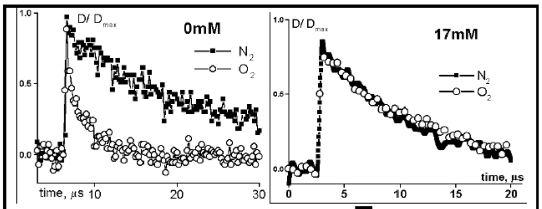


Figure 2-Effects of O_2 (○) and N_2 (■) atmospheres at different SDS concentrations (0mM and 17mM) on the lifetime of the X triplet state: 0mM: $\tau_T(N_2)$ = 11.2µs and $\tau_T(O_2)$=2.8µs; and 17mM: $\tau_T(N_2)$=8.3µs and $\tau(O_2)$=9.8µs.

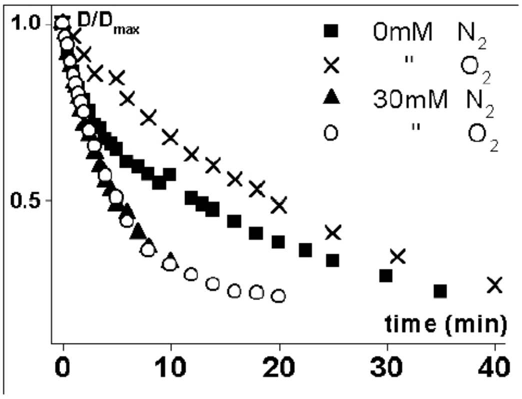


Figure 3- Effects of O_2 and N_2 with (30mM) and without SDS on the k_d rate constant of the X: ■: 10.4min; ×: 23.8min; ▲: 4.6min and ○: 4.7min.

In the presence of the surfactant τ_T and k_d do not depend practically on oxygen. At low surfactant concentrations (below CMC), the increase of SDS concentration produces the decrease of τ_T and the triplet state quantum yield (φ_T); and the increase of k_d until it reaches a maximum (near CMC). Further addition of surfactants (above CMC) increases τ_T and φ_T and decreases k_d (Figure 4).

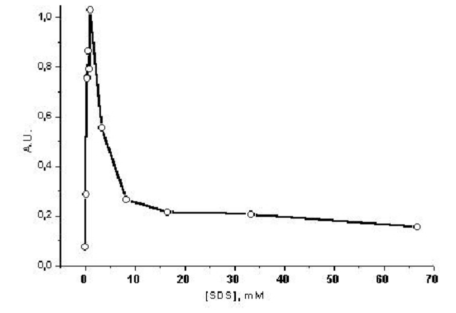
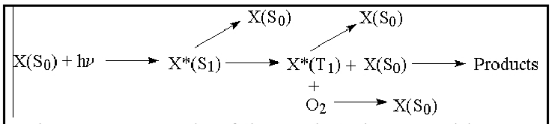


Figure 4- shows the photoreaction rate k_d dependence of X on function of surfactant (SDS) concentration.

Previously we have demonstrated [2] that photodecomposition of X in homogeneous aqueous solutions is in accordance with the scheme 1.



Scheme 1- Dynamic of the X photodecomposition.

The excitation by light produces the X singlet excited state [$X^*(S_1)$] which by intersystem crossing forms their triplet state [$X^*(T_1)$]. The reaction between the X molecule in its triplet state and an other one in the ground state [$X(S_0)$] is responsible for the X photodecomposition. Molecular oxygen quenches the X triplet state thus reducing k_d.

In the presence of low surfactant concentrations the mixed premicellar X-surfactant aggregates are formed which content more than one X molecule. These aggregates prevent X from the contact with oxygen and increase the probability of the contact between X in the ground and excited states and thus increase k_d. When the surfactant concentration increases the average number of X molecules in aggregates diminishes reducing the probability of this contact and thus reducing k_d.

Similar results were obtained [5] in the zwitterionic micellar solutions and at addition of bovine serum albumine (BSA).

Conclusion

The quinifuryl is effectively bound with micelles. This binding induces the aggregation of quinifuryl and protects their excited state against quenching by molecular oxygen thus stimulating their photodecomposition.

Acknowledgements

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PP-14 PVC PLASTIC BAG FOR LARGE-VOLUME PARENTERAL SOLUTION (LVPS): IS DEHP PRESENT ?

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Introduction

Large-volume parenteral solutions (LVPS) are commonly used to replace energy loss, to correct electrolyte disturbances in human body and also as a mean to provide basic nutrition. Recently, they have also been used as vehicles for drugs.

Plastics materials are widely used to pack LVPS and the most used forms are flasks or bags. The flexible plastic bag works physically by the forces of gravity and atmosphere pressure, and when the fluid leaves the container, the bag collapses. This collapse avoids outside air from entering the container and eliminates the possibility of airbone contamination. In addition, the possibility of air embolism occurring from this collapsible system is reduced.

Polyvinyl chloride (PVC) is the most used polymer for LVPS bags. In order to provide the necessary flexibility to PVC plastic bag, large amount of di-2(ethylhexyl) phthalate (DEHP) - a plasticizer - is used.

It is now known that DEHP can migrate from PVC bags into LVPS especially when the infusion contains hydrophobic drugs. For this reason, a lot of toxicological evaluation of phthalate plasticizers has been performed since it is evident that significant quantities may be inadvertently administered to the patient [2]. Though the influence of DEHP within the human body has not yet been made clear, the intake of DEHP into human body through bags to LVPS should be avoided, as recommended by World Health Organization [6].

The objective of this work was to verify the presence of DEHP in different commercial brands of LVPS package in PVC plastic bags market in Brazil using a gas chromatographic method.

Experimental methods

- **Materials**

Two commercial brands of LVPS which uses PVC plastic bags were supported by a grant.
Di (2-ethylhexyl) phthalate (DEHP) was purchase from Fluka, Switzerland.
η-Hexane and dichloromethane used was of chromatographic grade (MALLINCKRODT BAKER, Paris).

- **Chromatographic system**

Chromatographic analysis was carried out on a 14B Shimadzu Gas Chromatography equipped with a flame ionization detector (FID). A DB-5 (J&W Scientific, USA) capillary column, 60m x 0.25mm i.d. x 0.25µm film tickness was used. Hydrogen at the flow rate of 1.2ml/min was employed as carrier gas. Column temperature started at 140ºC, then was programmed at 10ºC/min. till 250ºC, then at 15ºC/min. till 280ºC and held for 25 minutes. Temperature of the injector port was held at 250ºC and the temperature of the detector was held at 300ºC. Nitrogen was used as make-up gas. The injector was operated in a split mode (1:20). Injection volume was 0.5µl.

- **Sample preparation**

PVC plastic bags were finely cut into 1cm^2 pieces which were submitted to extraction with 20ml of η-hexane or dichloromethane under ultrassonic bath (20 minutes). Samples were filtered through a Whatman paper nº1 and the volume was reduced to 10ml in a rotary evaporator. These solutions were diluted to 2:25 before injection into the GC-FID system.

Results and discussion

Figure 1 shows the chromatogram corresponding to a DEHP standard solution (concentration = 5µl/ml).

As it can be seen the retention time was 31.15minutes.

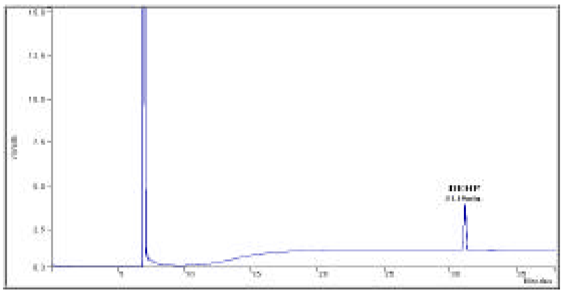

Figure 1. GC-FID chromatogram of DEHP standard solution in dichloromethane (concentration= 5µl/ml). Conditions: Column temperature: 140ºC to 250ºC at 10ºC/min, then 250ºC to 280ºC at 15ºC/min, held for 25 minutes: Carrier gas: Hydrogen; Flow rate: 1.2ml/min; Make-up: Nitrogen; Injection volume: 0.5µl; Injector port temperature: 250ºC; Detector temperature: 300ºC; Split mode: 1:20.

PVC bag extracts of the two commercial brands of LVPS also showed a 31.15 minutes peak, as shows the chromatogram of Figure 2, indicating the presence of DEHP. DEHP was also found in PVC bags used for LVPS, as described by AIGNASSE *et al*., 1995 [2] and by ABRANTES & DUARTE, 1992 [1].

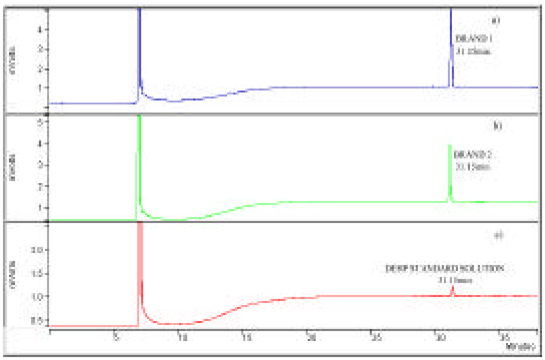

Figure 2. GC-FID chromatograms of DEHP exctracts from two commercial brands of LVPS which use PVC plastic bags, in dichloromethane: a)BRAND 1; b) BRAND 2; c) DEHP standard solution (concentration= 5µl/ml) used as reference. The same chromatographic conditions as Figure 1 was used.

Figure 3 shows the chromatogram corresponding of a dichloromethane and a η-hexane PVC extract of the same LVPS brand. The use of dichloromethane as additive polymer extractant has already been reported [3-5]. η-Hexane was also used to extract DEHP from PVC bags used to pack LVPS [2]. A clean chromatogram (Figure 2 and 3) was obtained, although PVC flexible bags usually contain a lot of additives.

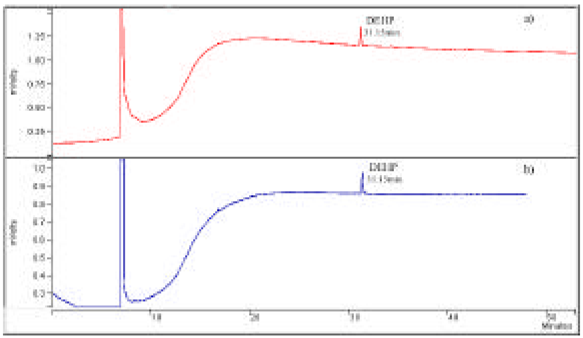

Figure 3. GC-FID chromatograms of DEHP extracts from one of the commercial brands of PVC plastic bags, using two solvents: a) dichloromethane; b)η-hexane.

The results obtained point out the importance of DEHP quantification in both PVC bags and LVPS in contact, in order to assess the potential health hazard presented to patients by exposure to DEHP.

Acknowledgements

This work was supported by Fapesp.

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PP-15 MOLECULAR SIMULATION STUDY OF STRUCTURE-FUNCTION RELATIONSHIP OF TWO PEPTIDES WITH ANTIGENIC PROPERTIES

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Introduction

Phospholipases A_2 (PLA_2S; EC 3.1.1.4) are abundant components of snake venom secretions. Its myotoxicity is one property in a diversity of toxic and pharamacological actions. Myotoxic PLA_2S from snake venoms are classified in groups I or II on the basis of their primary structure. The group II includes two categories where the residue 49 is Asp or Lys. These proteins are considered as to be homologue since they do not present catalytic activity on conventional substrates. Nevertheless, they exert myotoxic and membrane-damaging actions comparable to the wild type.

The myotoxin II is a highly basic, dimeric Lys49 PLA_2 isolated from the venom of *Bothrops asper*. The cationic/hydrophobic region including the 115-129 residues is involved in its cytolytic mechanism *in vitro* and in its bactericidal activity. Antibodies against peptide 115-129(p115-129) reduce the myonecrosis induced by the protein. The direct myotoxic effect of p115-129 peptide was not observed. The damaging action of p115-129 on membranes was proposed to depend on its amphiphilic character. This peptide contains three tyrosine residues surrounded by charged side chains that can establish interactions with membrane constituents. The hydrophobic residues seem to disturb the phospholipid bilayer integrity at the same time that the membrane interaction with the cationic residues stabilize the action of the hydrophobic residues. In order to test this model, one mutant peptide, similar to p115-129, was created substituing the tyrosine by tryptophan residues, (p115-W3). The amino acid sequence of these peptides are KKYRYYLKPLCKK (p115-129) and KKWRWWLKPLCKK (p115-W3). The peptide p115-W3 presents a drastic enhancement of the membrane-damaging activities together with a clear expression of myotoxic activity *in vivo*. The antigenic characteristics were conserved in p115-W3. Consequenly, p115-W3 reproduces all the known toxic effects of myotoxin II.

In view of the small size of p115-W3 in comparison with other bactericidal cationic peptides, its efficient activity is a strong suggestion that it can be a possible lead compound for the development of novel antimicrobial agents [1].

The structures of the p115-129 and p115-W3 were studied by molecular dynamics simulations since their understanding must clarify some features of their structure-activity relationship.

Experimental methods

The Newton’s equations of motion of all atoms in a model system are integrate numerically in molecular dynamics simulations. The molecular dynamics program was developed in our Group of Molecular Simulation (GSM) [2]. The system to be simulated consists of a solute molecule (peptide p115-129 or p115-W3) surrounded by about 3,000 water molecules that are maintained in a cubic box resulting in a water density equal to 49.0 mol . L^{-1}. The usual periodic boundary conditions and minimum image convention were applied. The GROMOS force field was used to describe the interaction potential energy for the solute and solvent molecules. The bond lengths were kept controlled using the SHAKE constraint algorithm as so as the bond angles in the solvent molecules. Experimental NMR data were used as initial p115-129. The initial coordinates for the p115-W3 were the same ones including the Tyr→Trp substitutions. The molecular dynamic simulations were conduced at 298 K during 1.5 ns after 0.15 ns to relax the system. The equations of motion were integrated using the Verlet algorithm with a 1.5 fs time step. A cut-off at 1.0nm and generalized Poisson-Boltzmann cavity field were used to take into account the long-range interactions [3].

The atom-atom pair radial distributions functions, g(r), the potential energy distribution functions, the radius of gyration, rmsd and distance between atoms were calculated during the simulations.

Results and discussion

At pH = 7.0, the p115-129 peptide contains 10 charged or polar residues including N and C-termini while p115-W3 contains 7 charged or polar residues. The interaction energy between p115-129 and the water molecules is smaller than in the p115-W3 case. Figure 1 shows the peptide-water interaction energy profile. From the beginning of the simulation, the energy becomes more and more attractive because more stable structures, the canonic structures, are found. The energy profiles are similar for p115-W3 and p115-129 until 0.1 ns while they converge after, at least, 1 ns. A hydrophobic cluster formed by a leucine and the three tryptophan residues was observed in the p115-W3 case. This cluster, located in the middle region of p115-W3, separates the charged and polar residues of the beginning from the end of the peptide. Consequently, both ends cannot interact directly. In p115-129, the Tyr residues form strong hydrogen bonds with Lys. This kind of interaction takes unfeasible the occurrence of many interactions between Tyr side chain atoms and water molecules. The lack of these interactions can be responsible for the p115-129 energy slow convergence.

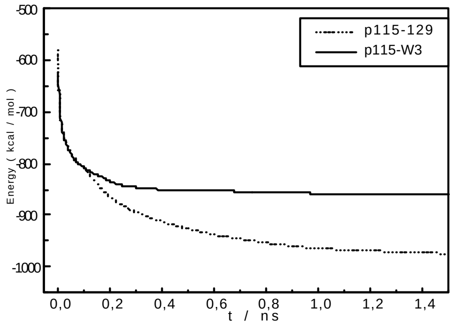

Figure 1. Energy of interaction peptide-water

Figure 2 shows the radii of gyration that consider only the atoms α carbon (CA) of backbone of peptides. The structure of p115-129 is more stable than the p115-W3 structure. The larger stabilization of p115-129 occurs because the tyrosine residues can be hydrogen bonded with Lys residues and with water molecules turning its structure more rigid than the p115-W3 structure. The hydrophobic cluster formed by triptophan residues confers more mobility in p115-W3 that p115-129. This mobility occurs because the flick of the triptophan residues is transferred by all peptide [2]. The radii of gyration of p115-W3 has values around 19 Å and its profile is more variable that p115-129.

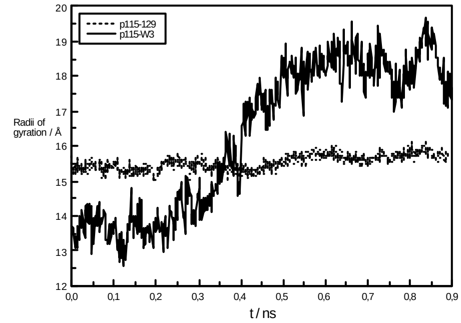

Figure 2. Radii of gyration considering only the α carbon atoms of the peptides backbone.

The figure 3 shows the structure of backbone of p115-129. It is a linear peptide and it agrees with the results of radii of gyration around of 15.5 Å. The figure 4 shows the structure of p115-W3 in the linear configuration with radii of gyration around 19 Å. The results show that the proposal of damaging action of p115-129 on membranes is in agreement with related results. The results include high mobility of p115-W3, a hydrophobic cluster and a structure more rigid that p115-W3. Each one of these characteristics indicate what are structural differences in p115-129 and p115-W3 that can explain their different actions.

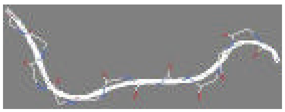

Figure 3. Structure of backbone of p115-129

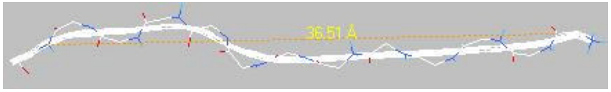

Figure 4. Structure of backbone p115-W3

Acknowledgements
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PP-16 SYNTHESIS OF AMPHIPHILIC TRI-ANTENNARY SACCHARIDE DERIVATIVES FOR TARGETED GENE DELIVERY

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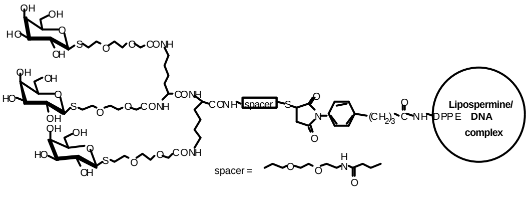
Introduction

Non-viral gene delivery systems, which are mostly based on the complexation and condensation of plasmid DNA by cationic polymers or amphipathic molecules have a promising potential. However, the overall positive charge of these particles which, in many cases, is necessary to observe appreciable transfection yields, singularly limits their application especially *in vivo*. Ligand-targeted gene delivery represents a mean to alleviate such limitations. It consists in using gene delivery constructs that carry at their surface ligands that interact with known receptors. The purpose is to replace the non-specific electrostatic interactions by a cell-specific one that triggers a receptor-mediated endocytosis of the targeted construct.

A particularly appealing approach for liver targeting consists in using the presence of the asialoglycoprotein or Gal/GalNAc receptor at the surface of hepatic cells to design vectors bearing glucidic residues. This receptor presents a great affinity for clusters of galactose [1,2]. Tri-antennary structures, with optimal intergalactose distances, are the best ligands for this receptor.

We have synthesized the tri-antennary galactose ligand Gal_3Lys_2 [3] presenting a good affinity for the Gal/GalNAc receptor. This ligand, conjugated at a lipidic anchor, was associated to a lipospermine/pCMV-Luc transfection particle (**I**). The presence of the ligand in the neuter lipoplexe increased the transfection yield [4]. This ligand however does not discriminate between the galactose receptor of hepatocytes and the lectin expressed at the surface of macrophages [5].

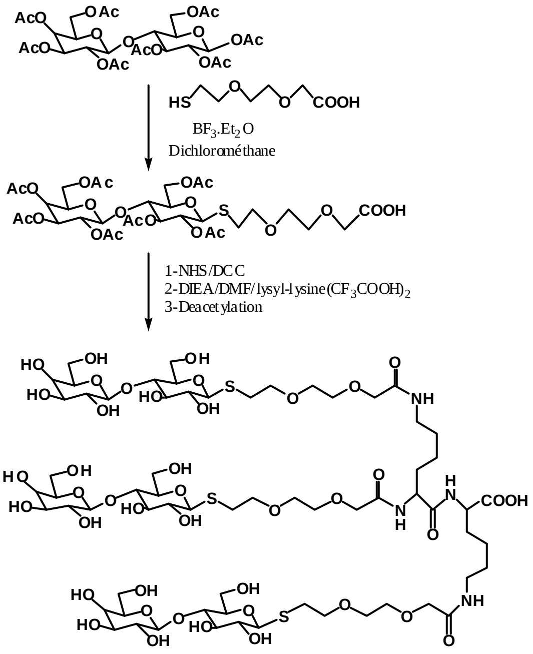


I

The goals of the present work were the synthesis of the new ligand Lac_3Lys_2 (**II**), which presents higher affinity for the Gal/GalNAc receptor of hepatocytes, and the synthesis of neolactolipids (**III, IV**) to prepared the targeted transfecting lipoplexes. These molecules carry spacers of different lengths for ligand/receptor accessibility studies.

Methodology

- **Synthesis of Lac_3Lys_2 (II):**

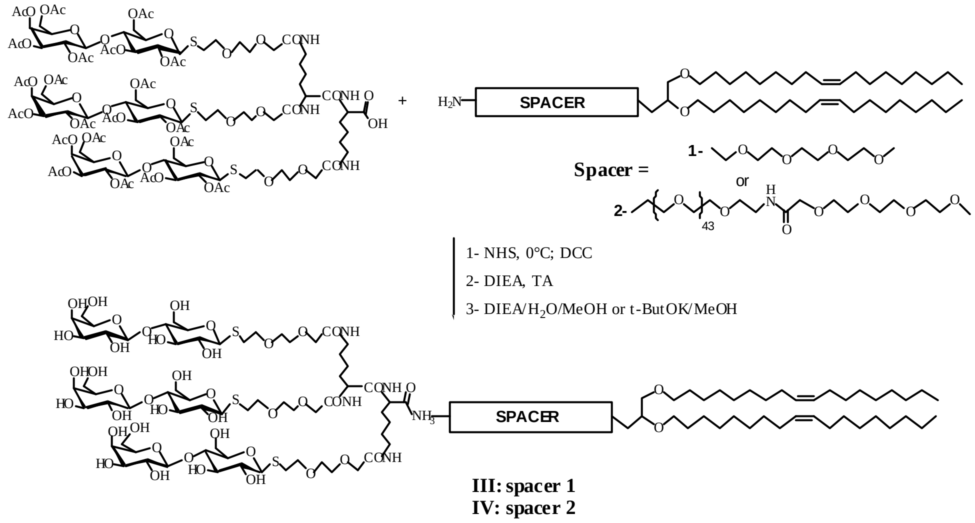


II

- **Binding affinity assay**

Gal_3Lys_2 and Lac_3Lys_2 affinity by ASGP-R of HepG2 cells (human hepatoma cells) was determined in a competition binding assay using the labeled ligand ASOR-FITC (asialo-orosomucoid-fluoresceinisothiocyanate) and increasing concentrations of Gal_3Lys_2 and Lac_3Lys_2, by flow cytofluorometric analysis. The competition binding data were analyzed with the Graph Pad Prism program.

- **Synthesis of neoglycolipids III and IV:**



- **Preparation of the lipid-pCMV-Luc complexes and cell transfection:**

Lipospermine (DOGS) was mixed with III at various proportions and with pCMV-Luc in 150 mM NaCl at 1 charge equivalent of DOGS/DNA. HepG2 cells were cultured in 24-well dishes, in DMEM. At 60%-confluence, cells were rinsed and transfection mixture was added. Fetal calf serum (10% v/v) was added after 3h and transgene expression was monitored 24h later. Luciferase activity was quantitated by photon counting.

Results

A) II was a white amorphous solid obtained with a 15% global yield. It was characterized by ^{1}H- and ^{13}C-NMR and by mass spectrometry.

B) IC50 of ligands:

Gal_3Lys_2: IC50 = 116,4 ± 41 µM
Lac_3Lys_2: IC50 = 0,28 ± 0,11µM

C) Compounds **III** and **IV** were yellowish amorphous solids, obtained with 30% and 25% of global yield, respectively, and both were characterized by ^{1}H- and ^{13}C-NMR and mass spectrometry.

D) Targeted transfection of HepG2 cells by [DOGS/neolactolipids/DNA] neutral complexes

- Complexes formed with 3, 5 and 10% of **III** yielded transfection increases of 140-, 120- and 90-fold, respectively, in comparison to non-targeted complexes.
- Complexes formed with **IV** didn't yield targeted transfection.

Conclusions

We have synthesized a tri-lactosylated ligand which presents a much higher affinity for Gal/GalNAc receptors of human hepatoma cells than its tri-galactosylated analogue. Two neolactolipidis were synthesized from this compound. Transfecting particles formed with 3-10% of III increased the transfection efficacy. This result could be interesting for use *in vivo*. Lipoplexes formed with compound IV didn't increase transfection; perhaps the large PEG spacer hampers the receptor-ligand interaction. The neolactolipids synthesized here could used be to target liposomes encapsulating drugs for the liver.

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PP-17 SYNTHESIS OF A NEW DRUG POLYMER CONJUGATE: POLY(PPG-CO-TARTARIC ACID-g- INDOMETHACIN)

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Introduction

In oral particulate delivery systems the drug is physically entrapped or adsorbed to the carrier. Drug loading of nanoparticles is conventionally via adsorption which is a variable process that also creates difficulties in separating free from adsorbed drug, so in some cases a suspension containing both free and adsorbed/entrapped drug has been used. A drug moiety can readily desorb from the nanoparticle due to, for example, changes in pH. An approach to promote oral absorption, enhance circulating half-life, or achieve drug targeting, is the formation of drug-polymer conjugate, e.g. conjugation of active drug moiety with polyethylene glycol or hydroxypropylmethacrylamide [1].

In this work we describe a novel approach that combines both method of oral absorption enhancement, i.e. the use particulated carriers and the use of drug-polymer conjugates, by synthesizing a drug-polymer conjugate that formed its own delivery system been formulated as nanoparticles. We have synthesized the poly(PPG-co-tartaric acid) (PPTA) which presents hydroxyl groups for the esterification with the carboxylic group of indomethacin, used as model drug.

Materials and Methods

- **Materials**

Indomethacin was obtained from Sigma (St Louis, USA. Poly(propylene glycol) (PPG, M_n = 425) was supplied by Fluka. Maleic anhydride (MA) was supplied by Carlos Erba (Porto Alegre, Brazil) and was used as received. All other chemicals reagents (as $KMnO_4$, HCl, NaOH) were reagent grade and used as purchased without purification.

- **Poly(PPG-co-maleic anhydride)** (PPMA)

The PPMA was synthesized by esterification of PPG and MA in bulk state, without any catalysts and by removing water. The synthesis was carried out at 70 °C during 4 hours, followed by 40 hours at 180 °C. The M_n of the polymer was 5,000 to 7,000.

- **Poly(PPG-co-tartaric acid)** (PPTA)

A 5 % (p/v) aqueous solution of $KMnO_4$ was dripped into a solution of PPMA in acetone until the medium became rose. Then, the MnO_2 was removed by filtration and the filtrate was evaporated by reduced pressure distillation. The resulting product was dried under vacuum at 40 °C during 1 day.

- **Poly(PPG-co-tartaric acid-g-indomethacin)** (PPTI)

The PPTI was synthesized by esterification of PPTA and indomethacin in bulk state. The polymer (PPTA) and the indomethacin were dissolved in acetone to homogenize the system. The reaction was carried out at 140 °C for 24 hours with the elimination of water and acetone. The PPTI was purified to eliminate free indomethacin. The polymer was dissolved in dichloromethane and the solution was extracted with 10 % (p/v) aqueous solution of $NaHCO_3$.

- **NMR analyses**

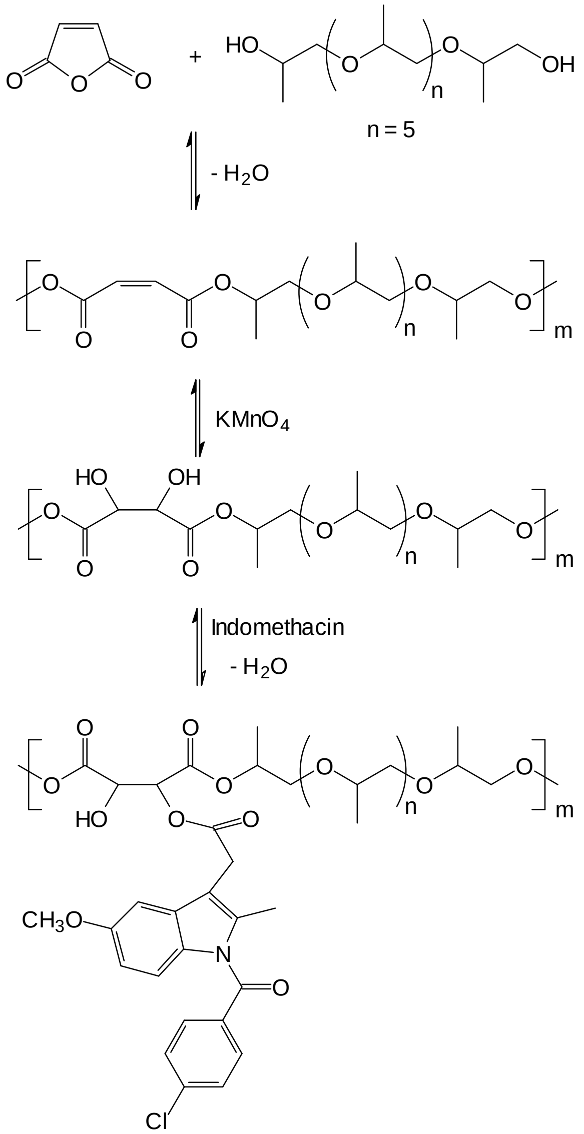
NMR spectra were recorded on a Varian VXR-200 spectrometer at a magnetic field of 4.7 T and at temperature of 22 ^{o}C. Chemical shift values are expressed as δ (parts per million) relative to TMS (internal standard).

- **Preparation of the nanosphere suspension**

The suspension was prepared by nanodispersion [2]. The organic solution consisted of PPTI (10.0 mg/mL), sorbitan monostearate and acetone. The organic solution was added under moderate magnetic stirring to the aqueous solution of polysorbate 80. The suspension was concentrated under reduced pressure for acetone elimination. The particle size was measured by dynamic light scattering at room temperature (20ºC) using a Brookheaven Instruments Goniometer BI200M/version 2.0 (200 mm) coupled to a BI9863 detection system. As light source a 35 mW Spectra Physics He-Ne Laser (model 127, λ_0 = 632.8 nm) was applied.

Results and Discussion

The polymeric synthesis using the monomers propylene glycol and maleic anhydride followed by the oxidation with $KMnO_4$ gave a product that presented solubility in water and high melting point, above 150°C. The reaction of this polymer and indomethacin in bulk state did not give the esterification product. On the other hand, the substitution of the propylene glycol by the poly(propylene glycol) (PM 425) in the reaction with maleic anhydride gave the PPMA (Scheme 1) that was oxidized with $KMnO_4$ furnishing the hydroxylated PPTA. The PPTA was soluble in acetone and presented its melting point at 120°C.



Scheme 1.

The reaction of PPTA with indomethacin gave the expected polymer (PPTI). The PPTI was characterized by ^{1}H NMR in $(CD_3)CO$ solution with internal standard TMS (Figure 1). The indomethacin moiety presented the chemical shift signals at 7.7 ppm (4H), 7.1 ppm (1H), 7.0 ppm (1H), 6.7 ppm (1H) for the aromatic protons and 3.8 ppm, 3.7 ppm and 2.3 ppm for the methoxyl, methylene and methyl groups. The PPTA moiety presented the chemical shift signals at 5.0 ppm, 3.4 ppm and 1.1 ppm for $O\underline{H}$, $OC\underline{H}$, $OC\underline{H}_2$ and $C\underline{H}_3$ groups.

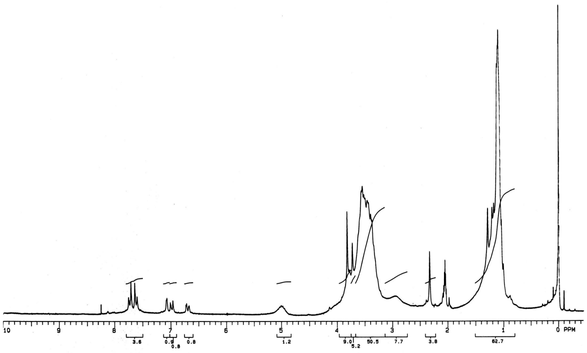


Figure 1. ^{1}H NMR of PPTI in $(CD_3)_2CO$.

Regarding the integrals, one aromatic proton of the indomethacin moiety for 63 protons of the methyl group of the PPG moiety showed that the esterification of indomethacin occurred in a proportion of 1:4 of PPTA hydroxyl groups.

Applying nanoprecipitation method, PPTI nanospheres were prepared. The light scattering measurements showed a monodispersed nanoparticles populations in the sub 400 nm range.

Conclusion

This work showed that poly(PPG-co-tartaric acid-g-indomethacin) were successfully synthesized. The results suggest that this new material can be used for the preparation of colloidal delivery systems.

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PP-18 STUDIES ON SPARTEINE AS CHIRAL INDUCTOR: MOLECULAR MODELING, KINETIC RESOLUTION AND ASYMMETRIC REDUCTION

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Introduction

Recently we carried out some studies on the asymmetric reduction [1,2], prevision of chiral induction by molecular modeling [3], etc..., correlated with the synthesis of optically pure ferrocenyl carbinols [4]. These carbinols are important as precursors for asymmetric catalysts [5,7] useful in the synthesis of optically pure pharmaceuticals [2]. Also, some ferrocenyl based new pharmaceuticals are known [1-7], as the ferrocenyl analogs of the chloroquine and tamoxifen. Due to this, molecular modeling of ferrocene based compounds is a interesting target for chemical and pharmaceutical studies.

In this work we now present the new results about:

a) The kinetic resolution of the racemic ferrocenyl methyl carbinol [4], mediated by a ruthenium complex of the natural alkaloid sparteine;
b) Prevision of the potential of some new ruthenium complexes with sparteine as chiral inductors, and sparteine complex with borane.
c) The asymmetric reduction of acetyl ferrocene [5] with borane/THF, with the chirality induced by valinol, leucinol and sparteine;

Methodology

Acetyl ferrocene [5] and methyl ferrocenyl carbinol [4] were prepared by previously published methods. (benzene)$RuCl_2$ dimer was prepared from 1,3-cyclooctadiene and ruthenium trichloride [6]. It reacts with sparteine in acetone as solvent, forming their sparteine adduct.

The reactions were carried out under inert atmosphere by Standard Schlenk techniques.

The calculations were carried out using the Chem 3D 6.0 software, MM2 method, in a Pentium 550, 160M Ram under Windows 98 as operational system [3].

The free base of the sparteine was liberated from the reaction of sparteine sulfate with barium hydroxide.

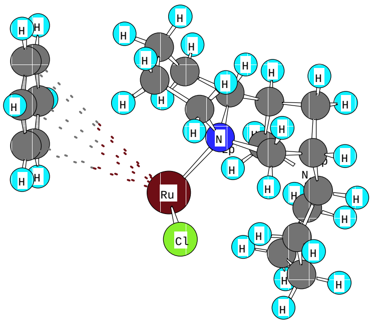
Results and Discussion.

We modified the procedure of Noyori [7], substituting their difficult to obtain ligand by sparteine. We carried out the kinetic resolution of racemic methyl ferrocenyl carbinol [4], using the complex formed from (benzene)ruthenium(II) dimer with sparteine as the chiral inductor, in acetone as solvent and hydrogen donor. We obtained the *l* alcohol, with 64% of enantiomeric excess and with 82% of optical purity.

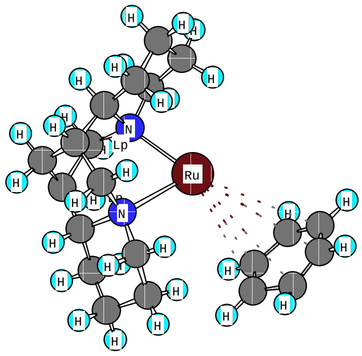
Molecular modeling was used as tool for the prevision of the chiral induction of the kinetic resolution by the (benzene)(sparteine)ruthenium complex.

The calculation of the adducts formed between sparteine and (benzene)ruthenium(II), showed that the open chain complexes (Figure 1) are more stable, but they may be much lesser effective than the chelated ruthenocycle (Figure 2). Furthermore, the results of optical purity and enantiomeric excess are satisfactory, even when the catalysis is carried out by one of the two possible open chain structures.

Studies on the isolation and caractherization of the (benzene)(sparteine)ruthenium complex were in progress.



146.39Kcal/mol
Figure 1- Complex Ru^{II} more stable

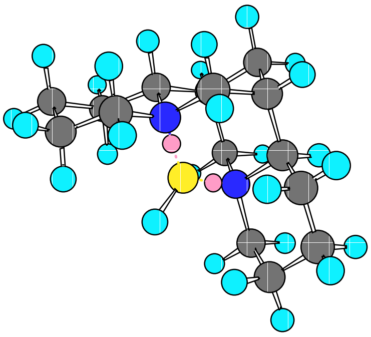


290.36 Kcal/mol
Figure 2 - Complex Ru^{II} less stable.

We reduced acetyl ferrocene by the modification of the CBS reduction employing borane/THF and valinol, leucinol or sparteine as chiral auxiliary. The chemical yields were high and the asymmetric induction as follows: Valinol: 35 % e.e. and 67 % o.p.; Leucinol: 42 % e.e. and 71 % o.p.; Sparteine: 65 % e.e. and 82 % o.p.

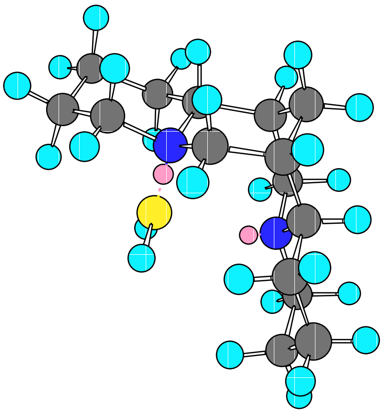
In order to understand if the structure of the sparteine-borane complex is flexible or not we performed several calculus of different structures looking for the most stable complex. We found that sparteine calculated was correct comparing to the literature [7].

The structure formed by the complexation of the borane to one nitrogen (Figure 4) of the sparteine is more stable than that formed by the complexation of the borane with both nitrogen (Figure 3). Although the complexation with both nitrogen makes the inductor more efficient, this structure is not the most stable complex.



76.41 Kcal/mol

Figure 3 - sparteine complex with both coord. nitrogens



51.91 Kcal/mol

Figure 4 - sparteine complex with one coord. nitrogen.

We observed, for the first time, that the reaction rate increases in the presence of excess of borane, which suggests that this kind of reaction may have second order kinetics.

Conclusion

Acetyl ferrocene can be reduced to the optically pure carbinol with satisfactory chemical yields, e.e. and o.p., by borane/THF in presence of valinol, leucinol or sparteine.

We concluded that the substitution of the difficult to obtain chiral inductor by sparteine in the kinetic resolution of the racemic methyl ferrocenyl carbinol gives the optically pure compound.

The reaction is mediated by a benzene ruthenium [8] adduct in moderate to high enantiomeric excess, but if the desired chelated ruthenocycle complex (sparteine chelate), (more unstable), would be the catalytic species, instead of the stable open chain complexes (real catalysts), the enantiomeric excess should be even greater.

Chiral ferrocenyl carbinols are important precursors for the preparation of chiral catalysts for the asymmetric synthesis of pharmaceuticals.

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PP-19 CHARACTERIZATION OF ISOLATED POLYMERIC FILMS: A STUDY OF THE SWELLING PROPERTIES AND THE WATER VAPOUR TRANSMISSIONS RATES

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Introduction

The use of polymeric membranes in pharmaceutical film coatings is widely used for the preparation of controlled release formulation. The film forming material used for theses purposes can be from natural, semi-synthetic or synthetic origin. The successful introduction of a wide variety of aqueous-based polymeric solutions and dispersions for film coating has stimulated research activity by pharmaceutical scientist and industry. The combination of differents material, which have film formed properties has been studied in previous papers [1, 4, 6]. Inulin carbohydrate is a naturally occurring gluco-fructan, which can resist hydrolysis and digestion in the upper gastro-intestinal tract. These oligosaccharides are found in much plant such as onion, garlic, artichoke and chicory. Chemically, inulin consists of a mixture of oligomers and polymers containing from 2 up to 60 or more D-fructose molecules, which are linked by β 2-1 bonds. In order to formulate inulin as a coating-material, it was incorporated as dispersion in cellulose for film formation, since inulin itself has no film-forming properties. Surelease® is a complete aqueous film-coating system, based on ethylcellulose, and formulated for use in controlled-release product application. The present study was to characterise the influence of addition inulin in differents polymers compositions (inulin – ethylcellulose) by studying their permeability (water vapour transmission - WVT) and swelling characteristics (hydration index) using isolated films of the polymers.

Experimental Methods

Material

Highly polymerised inulin (In) (Raftiline® from Orafti, Tiense Suiker, Tienen, Belgium), Simulated gastric (SGF) and simulated intestinal fluid (SIF) were prepared according to the *USP* 23rd edition (1995).

Methods

- **Film preparation**

Isolated films were prepared by casting process. Solutions (4% m/v) of inulin were mixed with Surelease® and stirred for approximately 5 hours. After complete homogenisation the dispersions were the remaining air evacuated using a vacuum pump. Samples of 10ml were poured over a nylon plate covered with Teflon® within circles previously demarcated. The nylon plates were stored in an air-circulated oven at 60°C for 10 hours, which corresponds with the minimum film formation temperature (MFT). The obtained films were carefully removed from the substrate and macroscopically examined for the presence of air bubbles and cracks, transparency and flexibility. Thickness was determined using a micrometer (Mitutoyo 2046F, Japan).

- **Determination of swelling index (Is %)**

Isolated films, corresponding to different compositions were cut in pieces of about 1 cm^2 and placed in a vacuum oven at 70ºC for complete drying during approximately 15 hours. The dried samples were stored in desiccator. Individual dry film pieces were weighed and immersed in different liquid media as simulated gastric (SGF) and intestinal fluid (SIF) for different periods of time. During the first 10 minutes the samples were removed every minute, dried between two filter paper sheets and weighed. After this period sampling was set at every 30 and 60 minutes.
The swelling behaviour of the films was quantified using equation (1), suggested by Blanchon et al. [2]:

$$Is\ (\%) = (Ms - Mi)/Mi \times 100 \qquad (1)$$

Where *Ms* is the film mass after a certain swelling period and *Mi* the dry film mass.

- **Water vapour transmission study (WVT)**

The study of WVT was carried out according to method B of ASTM designation E96-66 using Payne permeability cups (Braive Instruments, Liege, Belgium). Demineralized water (10mL) was put into the permeability cup and the cup was properly covered with the film closing completely the open side of the device. The cup with the film was weighed and stored in a desiccator filled with silica gel. After 1, 2, 3, 4 and 5 days of storage the cups were reweigh in order to determine the mass loss. The different values of mass loss were fitted to equation 2 and standardised to a 24 hours period of time, establishing the WVT for each polymeric composition tested [5]:

$$WVT = g\ 24/\ t\ a \qquad (2)$$

where g represents the mass loss, t the time measured in hours during which the weight loss was followed, and a the exposed area of the film, which was 10 cm^2.

Results and Discussion

A significant statistics difference was found between the values of swelling levels (Is%). These values were measured in order to determine the degree of hydration of the membranes. An increase in inulin concentration resulted in a statistically significant increase in degree of hydration of the films, which was the highest for the combinations: EC 80:20 IN and EC 90:10 IN, when compared against film control. Results of the swelling experiments are combined in Figures 1 and 2 for SGF and SIF, respectively. A supplementary rise in hydration was also observed when the films were immersed in the more neutral buffer solution, which simulates the intestinal fluid (SIF). This increase in hydration must be attributed to structural alterations of the oligosaccharide inulin. These results could be compared with data from Goycoolea *et al.* [5] who on evaluating the viscosity of polysaccharides under alkaline pH registered alterations of their behaviour in solution as steric expansion, and electrostatic repulsion between the constituent chains.

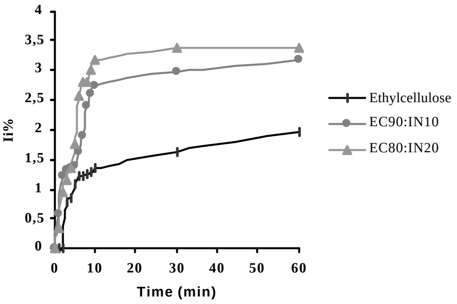

Figure 1: Swelling index in SGF (*p<0.05)

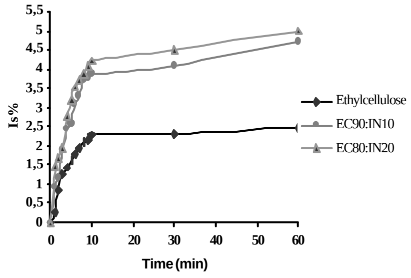

Figure 2: Swelling index in SIF(*p<0.05)

The increase of amount inulin addition on ethylcellulose films, allowed major permeability (WVT). It is apparent from Figure 3 that permeation of water vapour is affected by the composition. The WTV depends on the concentration of inulin in the membrane, which increase hydrophilicity. However, the analysis of variance (ANOVA), between the values of WVT, shown no one significant statistics difference relationship film control (ethylcellulose). Vervoort & Kinget [6] working in film formulation for coating showed that amount of incorporated inulin renders the films more permeable. Van den Mooter *et al.* [5] while working with azo polymers also observed an increase in water vapour transmission rate with increased hydrophilicity of these polymers.

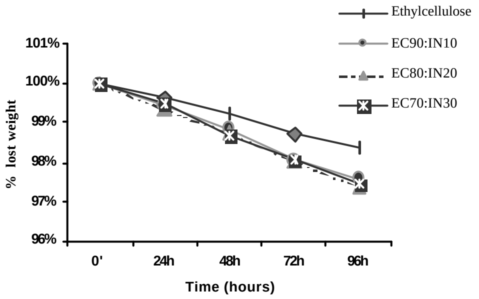

Figure 3: Water Vapour Transmission

Conclusions

The study has shown that inulin added permitted films to be obtained which resisted simulated gastric and intestinal fluid and maintain properties of membrane adequate for coating and favoured the degree of hydration (Is%) and WVT. These factors give us arguments to expect possible a new material coating added inulin for pharmaceutical use in controlled release system. The great merit of the associations of synthetic and natural polymers relapses over the perspective of remaining of their integrity through the gastrointestinal environment, due to their non-digestibility properties and potential material for site specific release.

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BD-01 IgM AND IgG GANGLIOSIDE ANTIBODY ASSAYS IN MICE TREATED WITH LIPOSOMES ASSOCIATED TO GANGLIOSIDES

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Introduction

Gangliosides are molecular structures composed of a hydrophobic tail (ceramide) and a hydrophilic (oligosacharide) moiety containing one or several residues of sialic acid head. They have functions as receptor or co-receptor for many bioactive agents such as cytokines, hormones, toxins and viruses. In addition they are considered to be involved in cell differentiation, morphogenesis, cellular recognition, cell-cell interaction and growth regulation [4]. Their pharmacological actions have been demonstrated on neural cells and tissues *in vitro* and *in vivo* on health animals and mostly with brain lesions of different nature, according to Pepeu et al. [11].

Since the identification of gangliosides as antigens presents in patients with neuropathy, the interest to study the pathological role of anti-ganglioside antibodies has been increasing. Mizutamari et al. [9], Latov [8] and Steck and Kuntzer [13] present some syndromes that may be associated to the anti-ganglioside antibodies. The high frequency of low titer of anti-ganglioside antibodies in neurological and non neurological diseases, as well as some normal controls has contributed to controversy about the pathological role of them [6, 9]. The side effects of gangliosides used therapeutically are not completely elucidated yet. It has been widely believed that protein-free gangliosides mixtures from bovine brain administered parenterally do not elicit immune-mediated side effects [2].

The association to liposome structures represents an alternative form for presentation of gangliosides to immunological system. The non-immunogenic and non-toxic characteristics of liposomes have been known from literature, resulting in wide applications as a drug delivery system. Otherwise, has been demonstrated that surface modified liposomes with gangliosides have a prolonged circulation time in blood stream compared to surface non-modified liposomes [7].

The aim of this work is evaluate the production of IgM and IgG antibodies to monosialoganglioside GM1 and to a mixture of gangliosides associated to liposomes, in health mice.

Material and Methods

Pure GM1 and a ganglioside mixture (Mix) composed by GM1 (21%), disialoganglioside, GD1a (40%) and GD1b (16%), trisialoganglioside, GT1b 19% were used for preparation of surface modified multilamellar liposomes (MLV). The lipid matrices were composed of dipalmitoylphosphatidylcholine (DPPC) and cholesterol (Chol). In some cases were added the dihexadecyl phosphate (DHP), a negatively charged lipid, which according to Allen [1] improve the uptake of conventional liposomes by the mononuclear phagocyte system (MPS). There were considered seven liposome compositions in this study: DPPC:Chol (60:40 %mol), DPPC:Chol:GM1 (40:40:20%mol), DPPC:Chol:GM1:DHP (40:35:20:5 %mol), DPPC:Chol:GM1:DHP (35:35:20:10 %mol), DPPC:Chol:Mix(40:40:20%mol), DPPC:Chol:Mix:DHP(40:35:20:5%mol), DPPC:Chol:Mix:DHP (35:35:20:10 %mol).

- **Animals**

Forty female Balb/c mice were used. Animals were housed in group of 4 per cage and fed standard chow and water *ad libitum*.

- **Liposome preparation**

The liposomes were produced by hydration of lipidic film according to New [10] and Beddu-Addo [3]. The liposomes were characterized by their phosphate contents by the method of Chen *et al.* [5] and the concentration of gangliosides was determined by sialic acid, through the colorimetry method of Svennerholm [14]. Liposomes presented a large size distribution with two main populations in which the major proportion presented a mean diameter 2000nm as measured by Photon Correlation Spectroscopy (PCS) at 90^0 C using a Malvern instrument (32 mW - He-Ne laser).

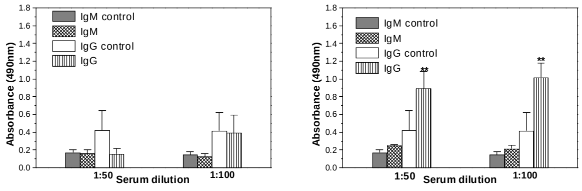
- **Immunological assays**

The gangliosides incorporated into liposomes were administrated by intraperitoneal (i.p.) route in mice in a total of six doses at two week intervals. Four mice were considered in each liposome evaluation and the control group did not receive any dose of liposome. The mice were bled from the heart and serum samples stored at -20^0C.

For the detection of antibodies against ganglioside was employed an enzyme-linked immunoabsorbent assay (ELISA) according to Ponzin et al. [12] with adaptations. Three concentrations of gangliosides were used to coating plates. 1:50 and 1:100 serum dilutions were used in the assays. The presence of IgM and IgG antibodies antigangliosides were considered positive only when the optical density, expressed as absorbance of the solutions at 490 nm, exceeded that of the mean value plus two standard deviations of control serum (cut off).

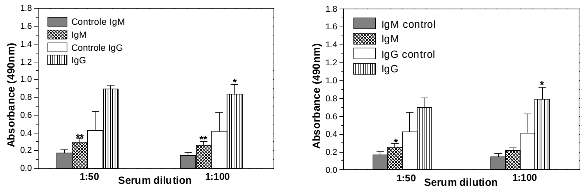
Results

The figures below show the production of antiganglioside antibody IgM and IgG compared to non treated health mice, used as control.



DPPC:Chol
(60:40 %mol)

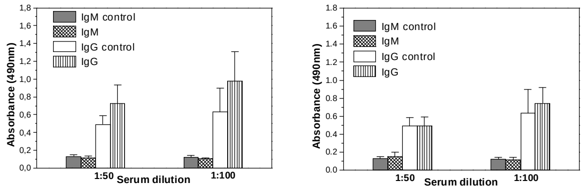
DPPC:Chol:GM1
(40:40:20 %mol)



DPPC:Chol:GM1:DHP
(40:35:20:5 %mol)

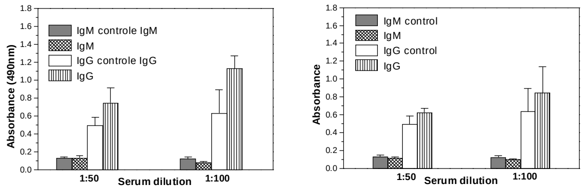
DPPC:Chol:GM1:DHP
(35:35:20:10 %mol)

Figure 1 – Antiganglioside antibodies of IgM and IgG class in plate covered with 10 μg/mL. *p<0.05 and **p<0.001 in relation to the control.



DPPC:Chol
(60:40 %mol)

DPPC:Chol:Mix
(40:40:20 %mol)



DPPC:Chol:Mix:DHP
(40:35:20:5 %mol)

DPPC:Chol:Mix :DHP
(35:35:20:10 %mol)

Figure 2 - Antiganglioside antibodies of IgM and IgG class in plate covered with 10 μg/mL.

Conclusions

Liposomes without gangliosides were non-immunogenic as well as that associated to the mixture of gangliosides, Mix, in all groups of studied mice. The GM1 incorporated into liposomes presented some immunogenicity, however, in relation to cut off considered in this study, the production of IgM and IgG class was negative.

From these results we conclude that ganglioside GM1 or the mixture (Mix) associated to multilamellar DPPC:Chol are potentially useful for immunotherapic applications, especially in cases where antibodies to gangliosides may have a pathogenic role.

Acknowledgments

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BD-02 ADSORPTION OF HUMAN IMMUNOGLOBULIN G USING L-HISTIDINE IMMOBILIZED IN METHACRYLATE MATRICES

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Introduction

Single amino acids have been reported to be fairly selective and efficient immobilized ligands for the purification of a variety of proteins. Previous work has shown that the amino acid histidine can be used as a ligand to specifically adsorb immunoglobulins of different sources [1].

It was shown that histidine could be coupled to sepharose in different ways, however sepharose-based supports have some disadvantages if the scale of the separation is to be increased. They are compressible and mass transfer is limited by diffusion [2].

As an alternative, this work focuses on the IgG adsorption onto metacrylate gels (Toyopearl) containing histidine as affinity ligand. These semi-rigid particles have the advantage of overcome the compressibility problem and can support moderate pressures drop caused by high flow rates.

The adsorption isotherms were constructed from the experimental data obtained in batch adsorption experiments at 25°C and 37°C. Adsorption isotherms were constructed at 37°C in order to verify whether the amount of IgG adsorbed in the gel would be different with temperature increases.

Experimental

- **Materials**

Gel Toyopearl HW-65F was purchased from Sigma (St.Louis, MO, USA). Human Immunoglobulin G was purchased from Centeon Pharma GmbH (Marburg, Germany).

- **Methods**

Immobilization of L-Histidine onto gel Toyopearl
The gel was activated with 1,4 butanodiol diglycidil ether and L-histidine was immobilized as described in protocols of Refs. [1,2].

Equilibrium binding analysis

The equilibrium binding experiments were carried out in batch mode at 25 °C and 37 °C for adsorbent with ligand. The data were analyzed using Langmuir, Freundlich and Langmuir-Freundlich models and non-linear regression computer program.

Results and discussion

- **Equilibrium analysis**

The interaction of human IgG with the His-Toyopearl gel was studied by chromatography and equilibrium binding analysis. The equilibrium adsorption data were analyzed using three different isotherm models, namely the general affinity interaction theory of Langmuir, the Freundlich model and the Langmuir-Freundlich model. This comparative analysis established the efficacy of the Langmuir-Freundlich model for describing the IgG binding to His-Toyopearl gel and explaining binding cooperativity and heterogeneity in quantitative terms.

The equilibrium adsorption data of IgG on the adsorbents is analyzed using the Langmuir-Freundlich model, described by the equation:

$$q = \frac{q_{m(LF)} \times (C_{eq})^n}{K_d + (C_{eq})^n}$$

where K_d is the apparent dissociation constant, $q_{m(LF)}$ is the maximum binding capacity, q is the amount of IgG adsorbed, C_{eq} is the equilibrium concentration and n is the Langmuir-Freundlich coefficient number. The isotherms and model parameters are presented respectively in Figure 1 and Table 1.

Table 1: Parameters to IgG adsorption onto derivatized Toyopearl at 25 °C and 37 °C

Parameters	25°C	37°C
Q_m (mg/mL)	23.96 ± 1.94	135.38 ± 179.44
K_d (M)	$1.90 \times 10^{-5} \pm 0.48 \times 10^{-5}$	$1.72 \times 10^{-4} \pm 1.8 \times 10^{-4}$
n	2.00 ± 0.47	1.07 ± 0.42
coefficient of correlation	0.992	0.986

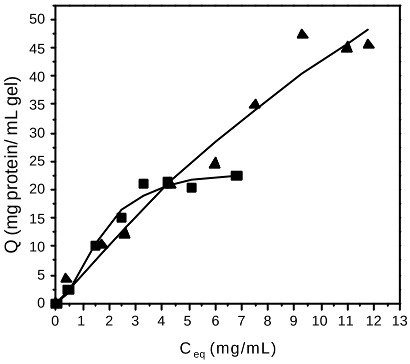

Figure 1: Isotherms at 25 °C (■) and at 37 °C (▲) for His-Toyopearl gel.

The isotherms presented in Fig. 1 shows that the model is able to take into account the sigmoidal shape of the experimental profile that arises mainly due to cooperative interactions and, thus, serves to describe adsorption cooperativity [3]. The amount of protein adsorbed in adsorbent at 37°C was significantly higher than that observed at 25°C, probably due the hydrophobic interactions, which are the most important for adsorption.

The Toyopearl polymer is rich in hydroxyl groups and ether bounds. The surface environment formed by Toyopearl polymer network is only mildly hydrophilic and may even display some hydrophobic tendencies under certain conditions [4].

The extent of nonspecific binding in our experiments is significant. The IgG molecules are adsorbed through nonspecific hydrophobic linkages or are retained inside particles of adsorbent.

- **Seletivity of adsorbents**

These experiments were run to demonstrate the adsorption selectivity of the Toyopearl and His-Toyopearl adsorbents for serum immunoglobulins. Untreated human serum was chromatographed on both adsorbents studied. Elution was performed with 0.4 M NaCl in the equilibration buffer. Both the non-retained and the pooled retained fractions of human serum were further analyzed using Bradford method. The amounts of adsorbed IgG on supports are given in Table 2. The SDS-PAGE (results not shown) indicated that the non-retained protein contains albumin, immunoglobulins and other serum proteins. The adsorbed proteins contain immunoglobulins with albumin and other protein as minor contaminants.

Table 2: Amount of proteins obtained in elution fractions for derivatized and underivatized gel

	Underivatized gel	Derivatized gel
Adsorbed proteins (mg protein/g dry suport)	0.34	0.65

Conclusion

The representation of experimental data by Langmuir-Freundlich model indicated the contribution of nonspecific interactions in IgG adsorption in this type polymer. The fact that the amount of protein adsorbed increased when the temperature was increased to 37°C, indicate the influence of hydrophobic interactions in nonspecific adsorption process.

Acknowledgements

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BD-03 FUCANS FROM THE BROWN ALGAE *Lobophora variegata* AND ITS ACTION ON THE REVERSE TRANSCRIPTASE

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Introduction

Fucans are a family of sulfated polysaccharides extracted from marine algae. These polysaccharides represent a source of marine compounds with potential applications in medicine. They are involved in several biological activities including antiviral, antithrombotic, anti-inflammatory, antitumoral and anticoagulant activity. This variety of functions is related to the great structural diversity presented in these compounds including chemical composition, conformation of the monosaccharides, quantity and position of the sulfate moiety in the polymer. In this paper we studied the chemical composition of this polysaccharide and the actions on trancriptase reverse from HIV.

Experimental methods

- **Materials**

Bovine serum albumin; α-(L)-fucose; D-galactose; D-xylose; glucuronic acid; D-mannose; toluidine blue, blue dextram 2,000 KDa, dextran 580 KDa, dextran 147 KDa and dextran 40 KDa, isobutiric acid, poly (rA), dATP, dCTP, dGTP, dTTP, DNase I, dithiotreytol were purchased from Sigma Chemical Co. Reverse transcriptase of HIV was obtained of the Boehringer Mannheim. (^{3}H)TTP was purchased from New England Nuclear (Du Pont). Agarose from Bio Rad Laboratories.

- **Extraction of the sulfated polysaccharides from the *Lobophora variegata***

The algae *L. variegata* dried powder from was suspended in two volumes of 0.15 M NaCl and the pH of the mixture was adjusted to 8.0 with NaOH. Fifteen milligrams of maxatase per gram of algae were added for proteolytic digestion, and the mixture reaction was incubated for 24 h at 60°C, followed by filtration through cheesecloth. The polysaccharides of the filtrate were further purified and fractionated by increasing concentration of acetone (0.3V, 0.5V, 0.8V, 1.0V, 1.5V, and 2.0V). In the study we focused the fraction F1.

- **Quantitation and identification of sugars and sulfate**

Fucose, xylose and uronic acid content of the fraction was estimated by the method described by Dische [1-3]. Total sugars were estimated by the phenol-sulfuric acid reaction [4]. Sulfate was measured after hydrolysis (8 N HCl, 100°C, 4 h) by the turbidimetric method [5]. The monosaccharidic composition of the fractions was determined by paper chromatography. The contamination with protein was measured by Bradford [6].

- **Electrophoresis**

Aliquots of the fractions (50μg) were applied in 0,6% agarose gel prepared with 0.05 M 1,3 diaminopropane acetate buffer, pH 9.0. The compounds were then fixed in the gel with 0.01% cetyltrimethylammonium bromide for 4 h. The gel was dried and stained with 0.01% toluidine blue.

- **Gel filtration**

Aliquots of fraction F1 (50mg) were applied to a column of Sepharose CL-4B. The column was developed with 0.2 M acetic acid. Total sugars were determined by phenol-sulfuric acid reaction [4] and uronic acid by the carbazole reaction [3].

- **Assay of effects of the F1 on the reverse transcriptase of HIV – 1**

The incubation medium was content: 50 mM Tris-HCl (pH 7.9), 40 mM KCl, 10 mM magnesium acetate, 2 mM dithiotreytol, 200 µg/ml of bovine serum albumin, 15 µg/ml actived DNA described by Aposhyan and Kornberg [7], 50 µM of each dATP, dCTP e dGTP, 10 µM (^{3}H)TTP (1000 cpm/pmol). The reagent mixture for the reverse transcriptase reaction was incubated at 37°C for 30 min. The reaction was stopped by the addition of 1 ml of 10% TCA cold. The mixture was filtered and the precipte on the nitrocellulose filter Milipore was washed with cold 5% TCA, containing 10mM sodium pirophosphate. The filters were dried and the radioactivity measured in a mixture of toluene/PPO/POPOP by scintillation. The incubation mixture consisted of 50 mM Tris-HCl (pH 7.9), 40 mM magnesium acetate, 2 mM dithiotreytol, 200 µg/ml bovine serum albumin, poly (rA)oligo $(dT)_{12}$ 0.24 A_{260} [poli A with absorbance of 0.2 at 260 nm and oligo (dT) with absorbance of 0.04] and 10 µM $(^3H)TTP$ (1000 cpm/ pmol) [8]. The reaction was initiated by the addition of 1 unit of reverse transcriptase, incubated at the 37°C for 30 min. The radioactivity was determined as described for actived DNA.

Results and Discussion

Fraction F1 showed a good degree of purification and two bands were evident after electrophoresis in agarose gel in 0,05M buffer diaminopropane acetate pH 9.0. Chemical analysis of F1 showed a molar ratio: 0.2 fucose, 1.0 xylose, 3.0 uronic acid, and 1.0 sulfate. This fraction has low levels of protein (4%) and high levels of polysaccharides (69.3%). The fraction F1inhibited the reverse transcriptase activity using two different substrates, activated DNA and poly(ra) $oligo(dt)_{12-18}$). This fraction inhibited reverse transcriptase by about 60.5% and 67.1%(for 0.5 and 1 mg respectively) using the activited DNA substrate and 87% and 94% using the poly(ra) $oligo(dt)_{12-18}$ substrate (Figure 1).

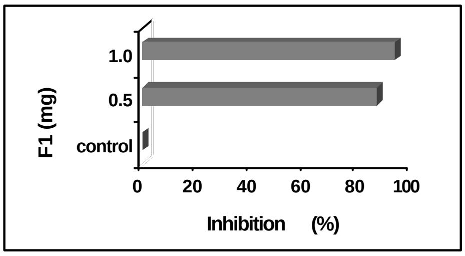

Figure 1 – Effect of the fraction F1 on the reverse transcriptase, utilizing substrate poly (rA)oligo $(dT)_{12-18}$.

Conclusions

The results suggest that F1 contains primarily sulphated polysaccharidics. This fraction is polidisperse in electrophoresis and has a high level inhibitory activity against the reverse transcriptase of HIV using to relevant substrates. These findings may foster the development of new anti-retroviral agents.

Acknowledgements

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BD-04 STUDY OF SERUM b - GLUCURONIDASE ACTIVITY: KINETIC PARAMETERS

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Introduction

Lysossomal β-glucuronidase (EC 3.2.1.31) is an essential catabolic enzyme that is involved in the degradation of sulfated glycosaminoglycans. Deficiency of β-glucuronidase in humans produces a mucopolysaccharide storage disease referred to as mucopolysaccharidosis type VII (Sly syndrome). In the absence of β-glucuronidase, chondroitin sulfate, dermatan sulfate and heparan sulfate are only partially degraded and accumulate in the lysosomes of many tissues. Mature β-glucuronidase is normally a homotetramer, but there is evidence that the homodimer can also be enzimatically active.

During the past few years, the measurement of serum and tissues markers has assumed a more significant role, influencing clinical decisions about treatment and follow up of patients with several diseases [1]. Hydrolytic enzymes are a major constituent of phagocytic cells, such as alveolar macrophages and polymorphonuclear neutrophils [2]. These cells have shown to be involved in many aspects of the inflammatory response and malignant diseases. Our interest has been focused on the relationships of use these glycosidases with gene therapy.

A major problem of tumor gene therapy is the low transduction efficiency of the currently available vectors. One possibility to improve the by stander effect could be the extra-cellular on version of a hydrophilic prodrug to a lipophilic, cell-permeable cytotoxic drug. It have used a secreted form of the normally lysossomal human β-glucuronidase to establish an extra-cellular cytotoxic effect system that converts an inactivated glucuronato derivative of drugs to cytotoxic drug [3]. The prodrug was originally designed to be activated into drug by human β-glucuronidase released from tumor cells in necrotic areas of tumor lesions. Most notably, this gene therapeutic strategy is shown to be clearly superior to conventional chemotherapy

In this study we investigated the activity of lysosomal glycosidase, serum β-glucuronidase, kinetic behaviour in healthy humans in comparison to the same enzyme in pathologic process.

Experimental Methods

- **Materials**

β-glucuronidase serum enzyme from healthy subjects, p-nitrophenol b-d-glucoronide (Sigma), bovine serum albumin (BSA), ascorbic acid, others analitical reagents.

- **Sample preparation**

The β-glucuronidase it was obtained from blood serum and kinetically characterized. To the blood was added NaCl 0.9% (1:6) and then centrifuged immediately. The supernatant rich in β-glucuronidase activity was utilized for the assays.

- **The b-glucoronidase enzimatic activity measurements**

The enzymatic activity it was measured at a 37°C, using a specific synthetical substrate 5mM p-nitrophenol-b-d-glucuronideo, 3.0mg of protein/mL suspended in 0.1 M acetate buffer pH 5.0, in a total volume of 1mL. The reaction was stopped 30 minutes after the enzyme extract addition with 1 mL of 0.5M NaOH. The production of p-nitrophenol was evidenced on 405 nm in alkalin medium. Protein level it was measured by Lowry method [4]. The kinetics characterization of the β-glucuronidase involved many aspects as the reaction time effect, optimum pH, cations effects, albumin, ascorbic acid and specific inhibitors.

Results and Discussion

The enzyme was stable for 40 minutes, at 37°C (figure 1). The optimum pH for catalysis was 5.0 in 100 mM sodium acetate buffer. The protein concentration in the sample was 3 mg/mL.

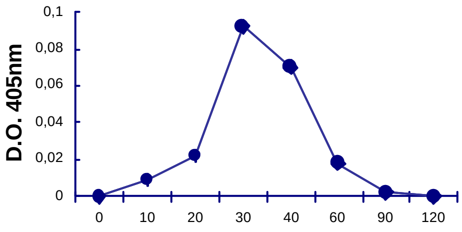

Figure 1–Thermal stability of enzimatic activity (min)

The enzyme showed high level activity when in presence of ascorbic acid at the concentration of 0.1%(figure 2). Ascorbic acid probably acts on the SH

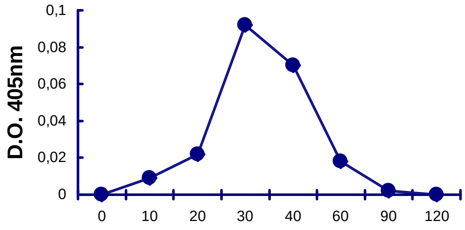

Figure 1–Thermal stability of enzimatic activity (min)

The enzyme showed high level activity when in presence of ascorbic acid at the concentration of 0.1% (figure 2). Ascorbic acid probably acts on the SH groups improving the enzyme conformation. Albumin not has any effect on this catalysis. This shows that interaction protein-protein does not change the conformation structural of enzyme that could act increasing or decreasing the catalysis velocity of the studied protein. The β-glucuronidase activity was changed by the presence of cations. The Hg almost completely inhibits the activity, which suggests that SH groups play an important role in the active site of the enzyme. When incubated with the inhibitor 1-4 lactona from sacharic acid, the β-glucuronidase was completely inhibited at the concentration of 4mM.

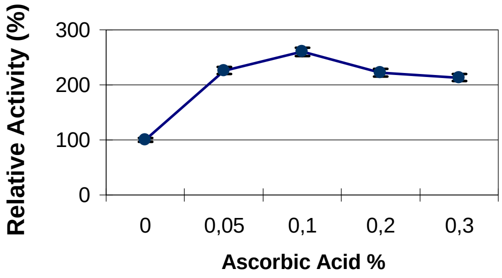

Figure 2 – Effect of ascorbic acid in the activity enzymatic

Conclusions

These results show that sodium acetate buffer pH 5.0 at 37 °C for 30 minutes was ideal catalysis condition for a β-glucuronidase. In presence of Ascorbic acid was observed an activation of the enzyme. Albumin not has influence on the catalytic reaction but this reaction it was affected by the cation presence and inhibited by Hg and activated by Cu and Mg. The β-glucuronidase was completely inhibited by the specific inhibitor 1-4 lactona sacaric acid a 40mM, showing clearly that the enzyme is a β-glucuronidase.

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BD-05 REVERSED MICELLAR EXTRACTION OF AN EXTRACELLULAR PROTEASE FROM FERMENTATION BROTH

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Introduction

Proteases are hydrolytic enzymes that have a large variety of applications, mainly in the detergent and food industries and are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds, etc. Proteases used in the food and detergent industries are prepared in bulk quantities and used as crude preparations, whereas those that are used in medicine are produced in small amount but require extensive purification before they can be used.

Microbial proteases account for approximately 40% of the total worldwide enzyme sales [1].

The extraction and purification of proteins using reversed micellar systems has been the subject of extensive studies in the recent years. Extraction using reversed micelles has the advantages of the liquid-liquid extraction technique and allows the solubilization of proteins in an organic phase by using surfactants, maintaining their functional properties. Reversed micelles are aggregates of surfactant molecules in the organic solvents. These surfactant aggregates consist of a polar inner core and an inner layer made of the surfactant hydrophilic head. The protein transfer can be influenced by: pH, ion strength, surfactant concentration, salt types and others [2].

The aim of this study was the extraction of an extracellular alkaline protease from an *Norcadiopsis* sp from fermentation broths using reversed micelles prepared in sodium di(2-ethylhexyl) sulphosuccinate (AOT) in isooctane .

Experimental methods

- **Microorganisms and enzyme production**

Norcadiopsis sp. isolated from soil in Pernambuco, Brazil, was grown in medium MS-2 [3] in orbital shaker (200 rpm) at 28°C for 48h. The cells (OD_{600}=1,0) were inoculated and cultivated using Erlenmeyer flasks (125 ml) containing 25 ml of the same medium, in the same conditions described before.

The alkaline protease is an extracellular enzyme and was produced during the stationary phase of the fermentation.

- **Forward transfer**

Reversed micellar solution was prepared by dissolving AOT in isooctane to the desired concentration. Equal volumes of two solutions, fermentation clear broth with 50mM KCl and reversed micellar solution were mixed by intense stirring for 10 min at 700rpm at 33^{0}C. The mixture was then centrifuged for 10 min at 3000xg, to phases separation.

- **Chemicals**

Sodium-di-2-ethylhexyl sulfosuccinate (AOT) was purchased from Sigma and Isooctane was obtained from Merck.

- **Protease activity**

Protease activity was measured spectrophotometrically, according to Leigton *et al*. [4].

- **Protein assays**

The protein in the aqueous phase and organic phase was measured using the spectophotometric assay of bicinchoninic acid (BCA), according to Smith *et al*. [5].

Results and discussion

- **Effect of AOT concentration on the extraction of alkaline protease**

Extraction studies of the alkaline protease directly from the fermentation broth were carried out with different AOT concentrations. The results presented in Figure 1 show the effect of AOT concentration (50-200mM) on the extraction of the protease from fermentation broth with 50 mM KCl at pH 8.0.

The percentage of total protein solubilized inside the reversed micellar phase increased with the increase in AOT concentration.

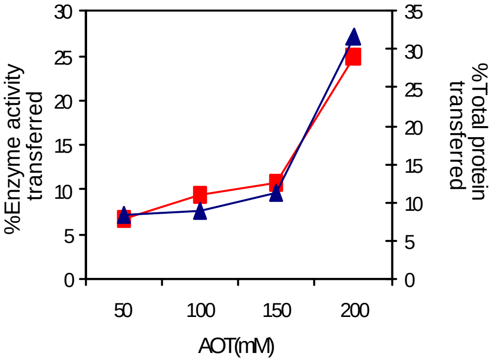

Figure 1. Effect of surfactant (AOT) concentration during forward transfer of the protease: (■) % enzyme activity transferred; (▲) % total protein transferred.

The best extraction was obtained using 200 mM AOT where about 31% total protein with 25% enzyme activity was transferred.

This behavior may be explained because the concentration of surfactant affects the size of reversed micelles, which may be attributed for increased transfer of total protein with the increase in AOT concentration [2].

- **Effect of ionic strength**

The effect of ionic strength (50-200mM) on forward transfer of protease was also studied as shown in figure 2. This was performed by contacting fermentation broth at pH 8.0 with 200 mM AOT in isooctane. The results show that the extraction of protease decreased significantly with the increase in ionic strength (Figure 2).

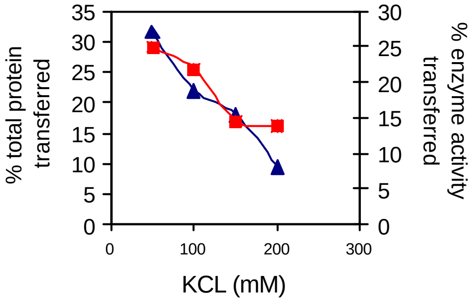

Figure 2. Effect of KCl on protease during forward transfer. ( )% enzyme activity transferred; ( )% total protein transferred.

The variation in ionic strength interferes with the size of the reversed micelles, selecting proteins according to their sizes.

Similar results for the effect of ionic strength in protein extraction in AOT/isooctane reversed micellar systems were also reported in the literature [6].

Conclusion

This studies show that the transfer of protease of the fermentation broth for reversed micelles is affected by the manipulation of parameters such as surfactant concentration and ionic strength. The best conditions achieved for extraction were: 200mM AOT/Isooctane with 50mM KCl at pH 8.0.

Acknowledgements

To Dr Ana Lúcia Figueiredo Porto give gently the *Nocardiopsi* sp culture, Laboratório de Imunopatologia Keizo-Asami (LIKA), PIBIC/CNPq and UFPE.

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BD-06 PRODUCTION OF L-ASPARAGINASE BY *Zymomonas mobilis* STRAIN CP4

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Introduction

L-Asparaginase (L-ASE) is an enzyme which has been applied as a potent anti-proliferative drug in neoplasic diseases, particularly in leukemias (specially lymphoblastic leukaemia). The anti-tumour activity of L-ASE has been known since 1960s [2] and is believed to result from rapid depletion of L-asparagine (L-Asn) in plasma, an amino acid essential to lymphoblastic leukaemia cells. L-ASE catalyses the hydrolysis of L-Asn into L-aspartic acid and ammonia.

There are two main sources of the enzyme, *Escherichia coli* and *Erwinia*. Most clinical studies employ the enzyme isolated from *Escherichia. coli*. There are two forms of L-Asparaginase (EC-1 e EC-2), but only EC-2 is effective in the treatment of leukemia. The two forms differ in solubility in ammonium sulphate solution, chromatographic properties, and effect of pH on enzyme activity. The enzyme from *E. coli* is commercially available however other sources of L-asparaginase are desired because of the incidence of immunological reactions in patients treated with the *E. coli* preparation.

Since the enzyme from different sources differ sensibly in their properties such as anticancer activity, immunogenicity, cytotoxicity, activity at physiological conditions, plasma clearance rate, substrate specificity, temperature, pH and salt tolerance etc., there is a great interest for L-asparaginase with properties suitable for its use as antileukemic agent. Its therapeutic and industrial importance demands not only the search for new and better yielding microbial strains but also economically viable bioprocess for its large scale productions.

Gulati et al. [4] developed a procedure for screening L-asparaginase producing microorganisms which gave a negative result for *Zymomonas mobilis.*

The objective of this work was to determine whether the bacteria *Zymomonas mobilis* CP4 produce a L-asparaginase enzyme and to study its production in a batch fermentation.

Experimental Methods

- **Bacterial strain and cultivation**

Zymomonas mobilis - CP4 was obtained from the collection of the Department of Antibiotics at the UFPE/BRAZIL The bacteria was grown in the minimal medium described by Alves [1], changing the nitrogen source to asparagine .

- **Fermentation**

Cells were growth at 33°C and 250 rpm on a bioreactor New Brunswick BIOFLO II. Growth was monitored by continuous measurement of optical density at 600nm in a Micronal 65B spectrophotometer. Glucose and ethanol concentrations were measured by HPLC technique.

- **Enzyme Activity Assay**

The L-asparaginase activity was indirectly determined through measurements of L-asparagine and aspartic acid in liquid chromatography. The enzyme was also detected by electrophoresis.

- **Electrophoresis**

L-asparaginase was detected in PAG gel electrophoresis as described by Manchenko. A solution containing L-asparagine (52mg), sodium tetraphenylborate (70mg) and 0.2M Tris-phosphate buffer, pH 8.0 (20mL) is mixtured with 20 mL 2% solution of agar and poured over the surface of electrophorized PAG. The gel is incubated at 37°C in a humid chamber until opaque bands appear.

- **Chromatography**

L-asparagine levels were measured using an HPLC technique after derivatization with o-phthaldialdehyde, as described by Puiatti and Sodek (1999), using isocratic separation instead of gradient separation. One minute after the derivatization, 20 μL were injected on to a Spherisorb ODS-2 (C18) (250 x 4 I.D. mm) column. The flow rate was 0.8 mL/min. The amino acids were detected with a fluorescence detector, using an excitation wavelength of 265 nm and emission cut-out filter of 480 nm. The mobile phase was the buffer consisting of 50 mM NaOAc, 50 mM Na_2HPO_4, 960 mL Milli-Q water, 20 mL tetrahydrafuran, 20 mL methanol. The pH was adjusted to 7.25 with acetic acid.

- **Preparation of samples**

The samples were prepared by adding 20 μL of OPA reagent to 60 μL volume of supernatants. The supernatants solutions were unfrozen only few minutes before the injection.

Results and discussion

The Figure 1 show the behavior of cells, glucose and ethanol concentrations for *Z. mobilis* batch fermentation. The cells concentrations were monitored continuously, while the glucose and ethanol concentrations were measured at discrete intervals.

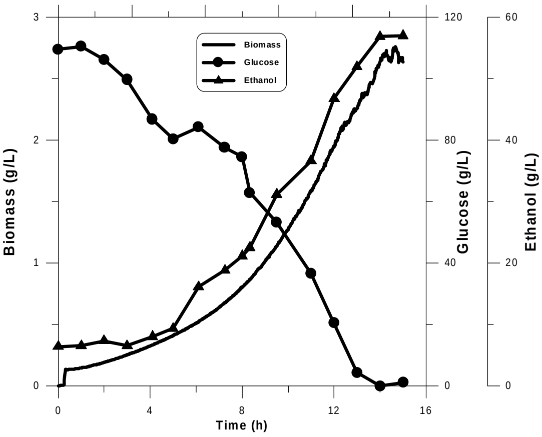

Figure 1 – Biomass, glucose and ethanol concentrations for a batch fermentation.

Figure 2 shown typical curves for L-asparagine and aspartic acid concentrations during the fermentation.

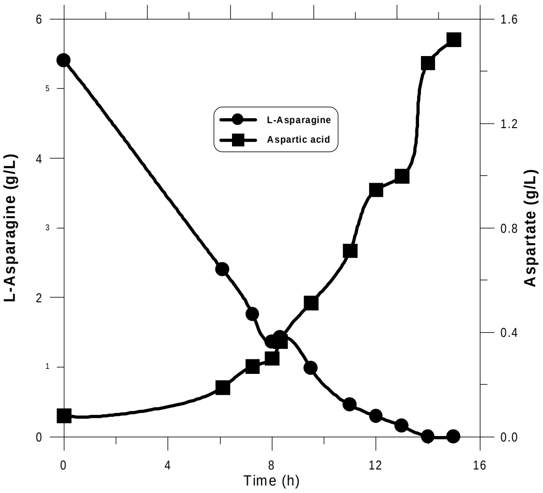

Figure 2 – Time profiles of L-asparagine and aspartate during fermentation by *Z. mobilis*.

The Figure 3 shows the opaque bands in the poliacrylamide gel (PAG). It was used 1mg/mL L-asparaginase from the medicine Elspar (Prodome) as standard. The standard was applied in the first two wells and in the two last. It was observed two opaque bands for the standard (L-asparaginase from *E. coli* – Elspar) and for the supernatant of fermented medium of *Zymomonas mobilis*. Its is known that there are two forms of L-Asparaginase (EC-1 e EC-2), but only EC-2 is effective in the treatment of leukaemia.

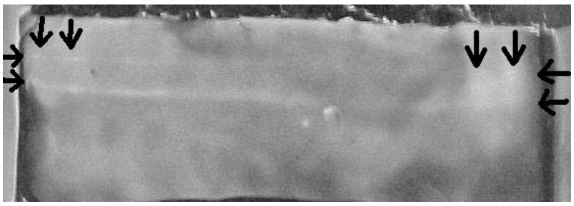

Figure 3 – Opaque bands (follow the arrows) resulting of the staining solution over the surface of electrophorized PAG.

Conclusion

It was reported for the first time that *Zymomonas mobilis* strain CP4 produces L-asparaginase enzyme. It was used gel eletrophoresis assay and monitoring of L-asparagine and aspartic acid by HPLC to confirm the enzyme production. The existence of two possible types of the enzyme was observed. The improvement of productivity e the verification of antileukaemic activity are further objectives of the study.

Acknowledgements

One of the authors (Pinheiro, I.O.) wish to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq for a scholarship.

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BD-07 ENZYMATIC PRODUCTION OF SORBITOL AND GLUCONIC ACID USING PERMEABILIZED *Zymomonas mobilis* CELLS

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Introduction

Sorbitol and gluconic acid are employed in several industries. Particularly, they have been widely applied in pharmaceutical and food industries. Sorbitol is an excipient for oral specialities of highest quality, food additive for dietary purposes, also used for: moisture conditioning and other wise improving quality of cosmetic cream and lotions, toothpaste, tobacco, gelatin; bodying agent for liquid pharmaceuticals and textiles; softener for candies; sugar crystallization inhibitor etc. Gluconic acid is not toxic, so several of its salts are employed in the pharmaceutical industries.

The conventional sorbitol production process is based on the hydrogenation, in the presence of nickel catalyst, of dextrose syrup at approximately 70 % concentration, and requires high pressure (40 atm) and high temperature (140°C). The residual Ni^{+2} in the syrup must be reduced to acceptable values in the food industry by passing through several ion exchange columns.

The gluconic acid can also be obtained by a chemical route, through the electrochemical oxidation of glucose in the presence of bromine ions. However, the conventional gluconic acid production process is based on a fermentative route, utilizing *Gluconobacter suboxydans* or *Aspergillus niger* in the oxidation of D-glucose.

There is a growing interest in the use of enzymatic processes for the production of several chemical processes in the pharmaceutical and food industries due to the relatively soft operation conditions demanded by these processes. Additionally the high specificity of the enzymes minimizes the formation of subproducts [1].

Glucose-fructose oxidoreductase enzyme (GFOR), present in cells of *Zymomonas mobilis*, is capable of converting glucose and fructose mixtures, oxidizing the first to gluconolactone and simultaneously reducing, the second to sorbitol. A NADP(H) cofactor is tightly coupled to the enzyme and acts as a hydrogen carrier. The produced gluconolactone is then hydrolyzed to gluconic acid by the action of the gluconolactonase (GL) enzyme, which is also present in the *Zymomonas mobilis* cells.

GFOR operates by a classic ping-pong mechanism in which the substrate consumption and product formation is equimolar. A unique active site is employed for the substrates (glucose and fructose) and products (gluconolactone and sorbitol), where each one is bound at a time, depending on the oxidation state of the enzyme-cofactor complex, which is regenerated after each reaction cycle [2].

Permeabilized *Zymomonas mobilis* cells allow the introduction of extrinsic molecules and the release of some compounds, while the GFOR and GL enzymes are retained. Besides improving the stability of the enzymatic process, as the enzymes are kept inside their natural environment, the process of production of sorbitol and gluconic acid by *Zymomonas mobilis* presents the advantage of eliminating the steps of isolation and purification of the enzymes, which reduces the costs of the process.

If intact cells of *Zymomonas mobilis* are employed, glucose and fructose substrates are converted to sorbitol, which accumulates in the medium, and to gluconic acid, which is converted by the Entner-Doudoroff route to ethanol and other low concentration subproducts. This mechanism is represented in Figure 1 [1].

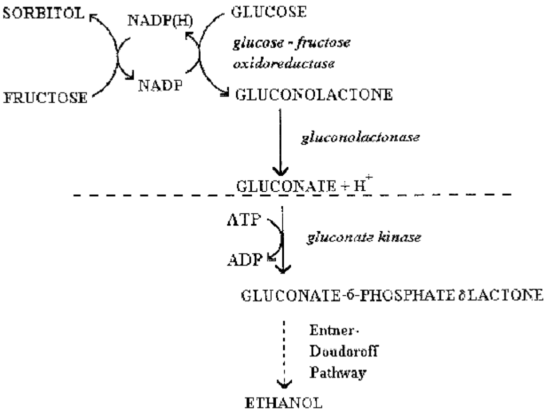

Figure 1 – Mechanism of sorbitol and gluconic acid production by the GFOR and GL enzymes and gluconic acid conversion to ethanol by the Entner-Doudoroth pathway.

The permeabilization of these cells acts in the removal of soluble cofactors and of high energy compounds which are necessary for the conversion of gluconic acid to ethanol by the Entner-Doudoroff pathway, avoiding the action of other enzymes involved in the metabolism of these sugars.

The goal of this work is to describe the main characteristics of the batch process for the production of sorbitol and gluconic acid using cells of *Zymomonas mobilis* permeabilized with cetyltrimethylammonium bromide (CTAB). The kinetics of the reaction is discussed.

Materials and Methods

- **Microorganism and Growth Conditions**

The experiments were performed with *Zymomonas mobilis* CP4 (ATCC 31821), cultivated anaerobically in a medium containing 100 g/L glucose and 5 g/L yeast extract, under controlled temperature of 30°C.

- **Cell Permeabilization**

In late exponential phase, after about 20 hours growth, CTAB was added to the broth in a proportion of 0.04 g per gram of cells [3]. After gently stirring for 30 min, cells were separated by centrifugation at 5500 rpm, washed twice with distilled water (pH 5.5) and resuspended in distilled water (pH 5.5).

- **Kinetic Studies**

Kinetic studies were carried out using different concentrations of glucose and fructose mixtures and free permeabilized cells. Reactions were performed in a glass bioreactor with a working volume of 50 mL. The temperature was controlled at 39°C and pH at 5.5 by a pHmeter (Chemcadec model 565200) that operates a peristaltic pump (Masterflex model 77120-60) for adding a solution of sodium hydroxide 0.3N to the medium.

- **Analytical Methods**

Cell concentration was determined as dry weight. The known concentration solution of NaOH neutralizes the gluconic acid formed in reaction. The mass of NaOH solution added to the system was measured during reaction course. In this way, substrate consumption and products formation was monitored on line, since this enzymatic reaction is equimolar.

Results

Several batch experiments were conducted, with all of them yielding high specific rates. A reaction was conducted with solutions of substrates at different proportions of glucose (100 g/L) and fructose (200 g/L). The initial cell concentration was 14.5 g/L. Figures 2 and 3 show the temporal evolution of the sodium gluconate concentration and the conversion, respectively. The initial specific rate of the reaction was 3.5 g gluconate/(g cell.h) and, as can be seen from Figure 3, about 94.3 % of conversion was reached in only approximatedly 4 hours of reaction.

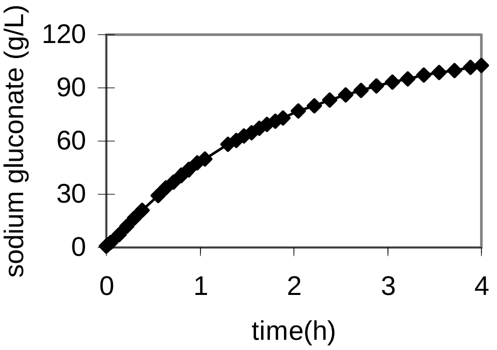

Figure 2 – Kinetic of sodium gluconate production, using a solution with different proportions of glucose (100 g/L) and fructose (200 g/L).

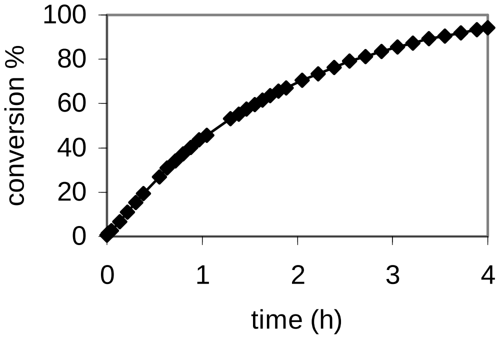

Figure 3 – Temporal conversion profile.

Conclusion

The enzymatic system, with permeabilized cells, is easily prepared, the kinetic rates are high and the enzymes works at very high concentrations of sugars and at amenable operating conditions (39°C, 1 atm, pH=5.5). These features provides a very attractive way to produce sorbitol and gluconic acid.

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BD-08 INFLUENCE OF PROCESS VARIABLES ON THE PRODUCTION OF VACCINE POLYSACCHARIDE BY *Neisseria Meningitidis* SEROGROUP C

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Introduction

Neisseria meningitidis (or meningoccocus) is one of the major pathogens that cause bacterial meningitis, which has been considered a worldwide health problem. Current vaccines for meningoccocal disease are composed by purified capsular polysaccharides that are immunogenic in adults but are not effective in children under two years old. However, polysaccharide-protein conjugated vaccines could be seen as a good alternative for protection of those infants. Therefore, in both cases, the production of capsular polysaccharide is extremely important and, consequently, its optimization can be viewed as an interesting goal to be achieved in the manufacture of meningoccocal vaccines [1, 2]. The objective of this work was to study the influence of some important variables (temperature, pH and aeration level of fermentation media) on the productivity of vaccine polysaccharide by *Neisseria meningitidis* C.

Experimental methods

The organism used was *Neisseria meningitidis* serogroup C 2135 from the Institute Merieux, France. *Neisseria meningitidis* has grown in Agar Müller Hinton 3.4% (w/v) plates at 37°C during 16 h in a candle jar (5-10% CO_2). Biomass content of plates was collected and transferred to 250 mL Erlenmeyer flasks with 50 mL of Frantz [3] medium and incubated in a shaker, 37°C, 200 rpm during 4h. These cultures were then used for inoculating 2L flasks with 450 mL of Frantz medium at the same growth conditions. The content of 2L shake flasks was used as inoculum (10% v/v) for experimental runs conducted utilizing 500 mL of Frantz medium in 1L vessel bioreactors (BIOSTAT Q, B.Braun Biotech International), which are equipped with sterilizable pH and dissolved oxygen electrodes, as well as temperature sensor and magnetic agitation speed controls. Culture medium samples were taken during 16 h of fermentation and assays for biomass, glucose and capsular polysaccharide concentration determination were done. Absorbance of the culture was measured at 600 nm using a Beckman DU-530 spectrophotometer and biomass concentration was determined by a linear correlation between dry cell weight and absorbance, calculated previously. Residual glucose profile was determined by enzymatic glucose-oxidase method (Bristol Diagnostic Kit #749). Capsular polysaccharide concentration was determined as sialic acid content using the colorimetric resorcinol method of Svennerholm [2]. Dissolved oxygen concentration was measured using a polarographic oxygen electrode. Global mass transport coefficient (k_La) was determined by gassing-out method [4].

Results and discussion

Actual production of C polysaccharide at LATEB (Bacterial Technology Laboratory of Bio-Manguinhos – FIOCRUZ) has been conducted in a 15 L bioreactor (BioFlo IV, New Brunswick Scientific Co.) with superficial aeration at 7.2 L/min air flow rate, being temperature controlled at 37ºC, initial pH adjusted at 7.4 (without pH control during fermentation course) and agitation frequency at 650 rpm. These conditions provided a k_La equal to 36 h^{-1}.

Initially, a scale-down schedule of the actual polysaccharide production process based on the global mass transport coefficient (k_La) was carried out. Four experiments under the same conditions of actual polysaccharide production – T = 37ºC, initial pH = 7,4 and k_La = 36 h^{-1} (upper aeration, agitation frequency = 1300 rpm; air flow rate = 0.8 L/min) were conducted. Figure 1 shows the average results attained by the scale-down procedure.

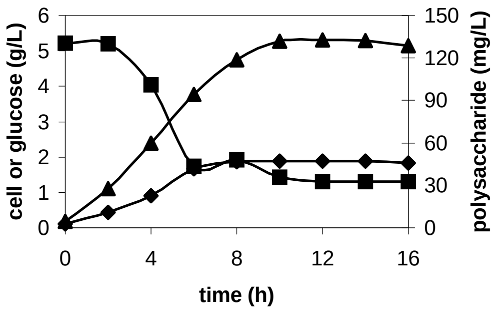

Figure 1 – Results for scale-down procedure

These results indicated that 15 L polysaccharide production had been reproduced in 1L vessels. Final average concentration of cell and polysaccharide for actual process were 1.7 g/L and 137 mg/L, respectively. These values when compared to those obtained for 1L fermentation – 1.9 ± 0.2 g/L and 131 ± 19 mg/L – could be assumed as statistically equal in a 95% confidence interval. In addition, biomass, glucose and polysaccharide concentration, pH and dissolved oxygen level profiles similarity reinforced the fact that the scale-down schedule was successfully achieved. This way, those four typical experiments could be used as a comparative parameter in the study of process variables influence on polysaccharide productivity. Seven experimental runs varying temperature, pH and agitation speed (aeration level) values under three different conditions were conducted in 1L vessels fermenter. Process conditions were made to vary near a considered central experiment (T=36ºC; pH=7.0 and agitation frequency = 1200 rpm or $k_La \approx 30h^{-1}$). The conditions of temperature, pH and agitation frequency (or k_La) tested were 33, 36 and 39ºC; 6, 7 and 8; and 900 (20 h^{-1}), 1200 (30 h^{-1}) and 1500 (40 h^{-1}) rpm, respectively. Figure 2 shows concentration profiles for central experiment.

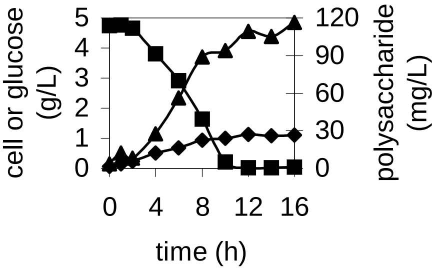

Figure 2 – Results for central experiments.

Some common characteristics could be visualized during the analysis of the experimental plan results, which indicated that pH control had probably induced some important modifications on the bacteria physiology. All seven runs achieved a stationary growth phase at 8 h. These experiments did not present residual glucose concentration as the usual experiment (Figure 1). Glucose had been totally consumed at about 8h in all experiments. In spite of stationary growth phase and total glucose consumption after 8h, increasing polysaccharide concentration continued to be observed. This fact indicated that the production of capsular polysaccharide by *N. meningitidis* is only semi-associated to bacterial growth.

Analysis of dissolved oxygen profiles indicate that oxygen concentration in fermentation medium reached extremely low values (approximately zero) at about 2 h. A correlation between oxygen consumption and polysaccharide formation could be supposed, since polysaccharide stationary phase were only observed when dissolved oxygen concentration turned to increase. These observations showed that even after stationary growth and glucose consumptions phases, cells continued active and producing capsular polysaccharide. Based on experimental results, optimal values for process variables were evaluated, which are T=37ºC, pH=7.1 and agitation frequency = 1,130 rpm (k_La=29 h^{-1}). The results for optimal conditions analysis can be viewed in Figure 3. None of seven experimental runs presented final concentration values greater than those obtained from usual experiments (Figure 1). However, central experiment (T=36ºC, pH=7.0 and agitation frequency = 1,200 rpm), which is very closed to the proposed optimal experiment, presented a final polysaccharide concentration value (P_f = 116 mg/L) that could not be considered statistically different from the average value of usual experiments (P_f = 131 ± 19 mg/L). This way, new experiments should be conducted for evaluating final polysaccharide concentration at proposed optimal conditions.

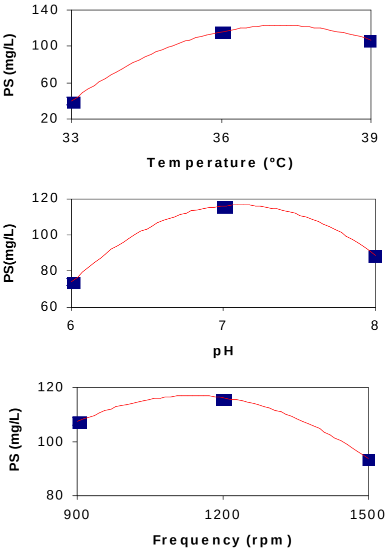

Figure 3 – Effects of process variables on polysaccharide production

Conclusion

At this work the influence of temperature, pH control and agitation frequency (aeration level) on the production of vaccine polysaccharide by *N. meningitidis* C was studied. Optimal values for these process variables were proposed. Although pH control induced changes on fermentation profiles, the best final polysaccharide concentration values obtained were similar to those attained in experiments without pH control. This fact indicated that pH control provides polysaccharide production results at least similar to those usually obtained. These results should be validated, since pH control will be necessary for testing others bioreactor operation modes.

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BD-09 FLAVONOL GLYCOSIDE PRODUCTION IN INTACT PLANT AND CALLUS CULTURES OF *Gomphrena globosa*

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Introduction

Many medicinal compounds are produced from the secondary metabolites of plants. Clearly, the development of alternative and complementary extraction methods for the production of clinical medicines from the whole plant is an issue of considerable socioeconomic importance. The recent use of plant cell culture technologies has been an alternative for the production of pharmaceuticals.

Some Brazilian *Gomphrena* species are employed in the treatment of bronchial affections, diarrhea and fever, and as an analgesic, tonic or carminative [1-3]. The aqueous extract of *G. globosa* presented antitumoral activity [4]. Preliminary studies with callus culture of *G. globosa* demonstrated the presence of pigments [5] and the activity of phenylalanine ammonia lyase (PAL) [6] were also detected.

Goal

The aim of this work was to verify the presence of secondary metabolites from intact plant in cell culture of *G. globosa*.

Methodology

Aerial parts of the *G. globosa* were collected in March 1995 and identified by Dr. Josafá Carlos de Siqueira. A voucher specimen (SCAB 4050) was deposited in the herbarium of Nova Friburgo, Rio de Janeiro.

Dried powdered plant was extracted with ethanol and partitioned with hexane and dichloromethane, successively. The methanol: water phase was concentrated and partitioned in an *n*-butanol-water mixture. Extracts were dried under vacuum in a rotatory evaporator. The water insoluble portion of the *n*-butanol layer was chromatographed on Sephadex LH 20 and a few mg of the pure flavonol glycoside were isolated.

Leaves from plantlets of *G. globosa* were washed in tap fresh water for 24 h, treated with 1% Benomil™ for 40 min and sterilized by stirring in 0.5% sodium hypochlorite solution for 20min, followed by three successive rinses in sterile distilled water. Leaf explants were inoculated in the basal medium of Murashige and Skoog [7] supplemented with phytohormones. The hormone concentrations employed were T43 (1.0mg/L 2,4-D and 1.0mg/L kinetine) and T11 (2.5mg/L NAA and 1.0mg/L BA). The callus culture was maintained under the followed conditions, photoperiod of 16 h and temperature of 25±2ºC. After 30 days of cultivation the callus culture was dried at 60ºC and extracted with chloroform and methanol. Both extracts were grouped and submitted to partition with hexane and chloroform. Extracts were dried under vacuum in a rotatory evaporator. The aqueous phase was lyophilized and fractionated on Sephadex LH-20 furnishing the same glycoside flavonol obtained from the intact plant.

TLC using the following mobile-phase performed the comparative study of metabolites production: chloroform, methanol and *n*-butanol (5:4:1)

Results

The *n*-butanol soluble portion of the ethanol extract of aerial parts of *G. globosa* was fractionated with on Sephadex LH-20 column. The compound isolated was supported by UV, ^{1}H NMR and ^{13}C NMR spectra and identified as *4',5-dihydroxy-6,7-metilene dioxyflavonol 3-O-b-D-glucoside*.

The grown of callus culture was not affected by phytohormones, but the difference of these modified the metabolites production. Betalains production was accumulated in the T11 media culture meanwhile not one pigments was seeing in the T43 media culture. Expression of secondary metabolism maybe regarded as a result of a degree of differentiation.

The comparative study on TLC was performed with Sephadex LH-20 column fractions of callus culture extracts of *G. globosa*. This study showed the presence of the flavonoid glycoside, confirmed by NP-PEG reagent [8].

Conclusion

In this study was possible to verify that cell cultures of *G. globosa* are able to produce a variability of compounds such as flavonoids. The production of metabolites in cell culture can be improved through biotechnology manipulations such as: biotic and abiotic elicitors, UV irradiation and others. Thus plant cell culture technology can be an important tool for chemistry and pharmaceutical industries in the production of bioactive compounds.

Acknowledgements

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BD-10 INTERACTION BETWEEN METHYLENE BLUE AND CARBODIIMIDE-MODIFIED HEPARIN

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Introduction

Biological tissues have been used extensively for manufacturing bioprostheses such as prosthetic heart valves [1], pericardial patches [2] and vascular grafts [3]. The ability of surface-immobilized heparin to prevent material –induced thrombus formation is well known. Hence, to overcome the thrombogenic problem commonly observed in vascular grafts, heparin have been immobilized on surface of these bioprostheses. Water-soluble carbodiimides have been chose to attach the heparin on these bioprostheses.

Cationic dyes such as alcian blue, toluidine blue, methylene blue and azure A have been used to determine glycosaminoglycans [4–7]. Methylene blue (MB) has been used as a staining reagent in the determination of heparin [7,8]. The method is based on the different absorption spectra of dye and dye-heparin complexes.

The purpose of this study was to observe whether the modification of the heparin by carbodiimide could change your interaction with methylene blue and consequently suggest the activation of the heparin.

Materials and Methods

- **Materials**

Heparin (Liquemine) was obtained from Roche Laboratories. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was purchased from Sigma. Methylene blue from Reagen. All other reagents were analytical grade.

- **Preparation of carbodiimide-modified heparin**

The methodology used consisted in dissolve the heparin in distilled water under agitation, and to adjust the pH to 4.7 with hydrochloric acid. After that, carbodiimide solutions (4 – 10mg/mL) were added and the pH controlled at a range of 4.5 to 5.0 for 1 hour. Then the pH was adjusted to 8.0 with sodium hydroxide solution.

- **Assay of the interaction between methylene blue and carbodiimide-modified heparin**

This experiment was carried out according Smith et al. (1980) [9] with some modifications, such as: methylene blue instead of toluidine blue; samples were not extracted in hexane.
Standard curve of heparin (10 – 50 μg/mL) was constructed using a methylene blue solution (0.005% in 0.01 N HCl containing 0.2% NaCl) at 664 nm. In this curve the absorbance decrease with the increasing of the heparin concentration. Thus samples of the carbodiimide modified heparin were analysed with a view to observe the interaction of these with methylene blue.

Results and discussion

Methylene blue (MB) dye binding heparin is not affected by the pH at a range of 2 to 12. Dye binding requires a macromolecular form with both carboxyl and sulfate-rich groups.

The heparin determination method of dye-heparin assay is simple because it is rapid, sensitive and relatively inexpensive, however, a little effort has been made to investigate the mechanism of the methylene blue dye binding and also to identify the functional groups involved in that linkage [10].

Figure 1 shows that the increasing of absorbance is related with the increasing of carbodiimide concentrations. Thus, it is observed that the carbodiimide-heparin activated did not show the same interaction with methylene blue as the single heparin (3.3mg/mL; Absorbance 0.713). This suggest that the carboxyl groups are important for interaction between heparin and methylene blue or there is a steric impedance. The figure shows a linear behavior between carbodiimide-modified heparin and absorbance at 664 nm, with a correlation coefficient of 0.99.

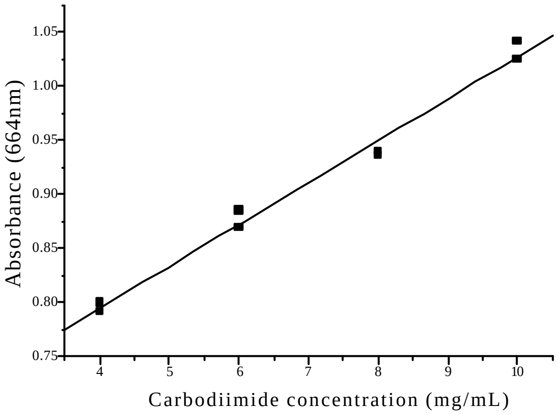

Figure 1. Effect of carbodiimide concentrations on the heparin solutions (3.3mg/mL) activated by using that agent.

Conclusion

This work showed that the modification of the heparin by carbodiimide interfere on the complexation of this glycosaminoglycan with methylene blue, which is depending on the concentration of carbodiimide. Hence, the quantification of the heparin treated with carbodiimide by methylene blue method will not provide a real amount of the heparin. However, the correction of the absorbance data may permit the quantification of modified heparin.

Acknowledgments

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BD-11 ADSORPTION MECHANISM OF ALKALINE PHOSPHATASE AT AIR-WATER INTERFACE AND ON PHOSPHOLIPID LB FILMS IN THE PRESENCE OF A NONIONIC SURFACTANT

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Introduction

Several aspects of biological membranes can be better understood from the study of protein adsorption at interfaces. Also the immobilization of enzymes can be used as biosensors as well as bioreactors. Several methodologies were developed in order to get the immobilization of enzymes and at the same time keeping their catalytic activity. The Langmuir-Blodgett technique allows the preparation of organized thin organic films (LB films) through the deposition of a controlled number of layers from monomolecular films formed at liquid/air interface. LB films formed by lipid layers can be a good matrix to adsorb proteins.

Alkaline phosphatase is a enzyme responsible for the catalytic action of the phosphoric esters hydrolysis. It can be obtained from rat osseous plate One of its forms behaves as an insoluble amphiphilic molecule. It is bound to the biological membrane by a hydrophobic anchor of phosphatidyl-inositol. The extraction of this enzyme makes use of a nonionic surfactant that makes it soluble in the aqueous medium and does not destroy its catalytic activity.

The objective of this work was to study the adsorption of alkaline phosphatase (AF) solubilized with a nonionic surfactant, poly(oxyethylene-9) lauryl ether ($C_{12}(EO)_9$), at the air-water interface and on DMPA-Zn^{2+} LB films and also to verify the influence of the surfactant on the adsorption mechanism.

Experimental methods

· Extraction and purification of alkaline phosphatase

Rat osseous plate alkaline phosphatase was prepared and purified according to the procedure described by Curti et al. [1] and its solubilization with $C_{12}(EO)_9$ and further purification was performed according to Ciancaglini et al. [2].

• LB films preparation and adsorption studies

DMPA-Zn^{2+} LB films were prepared by the transfer of DMPA (dimyristoyl phosphatidic acid) monolayers formed on zinc acetate solutions (1.10^{-4} mol/L), maintained at a constant surface pressure of (30±1)mN/m at (23±1)^{o}C. These monolayers were prepared in a homemade Langmuir trough by the spreading of a DMPA solution in chloroform (Carlo Erba) and methanol (Merck) (3:1). In all the cases, five layers were deposited in a “Y” type film, ending with the hydrophobic tail of the phospholipid. The kinetics of adsorption, as well as the adsorption isotherms, of the alkaline phosphatase on DMPA-Zn^{2+} LB films were carried on quartz crystals (ICM-USA) covered with gold electrodes. The quartz crystal microbalance (QCM) technique was used to detect the adsorbed amount of protein by measuring the decreasing in frequency (frequencymeter - Minipa- MF 610). The kinetics of adsorption studies were accomplished by QCM measurements changing the permanence time of the crystal into the protein solution. After each five minutes the crystal was withdrawn from the enzyme solution at a velocity of 0.1mm/s, using an electro-mechanic device. The first measurement was recorded just immersing the crystal and taken it out immediately, in such way the crystal with the DMPA-Zn^{2+} layers remain in contact with the protein solution for no more than 10 seconds.

After each withdrawn the crystal was dried as far as no frequency variation was detected, that is, until constant mass is achieved.

Three concentrations of $C_{12}(EO)_9$ were used for protein solubilization: 0.005%, 0.01% and 0.05%.

• Critical micelar concentration (CMC) $C_{12}(EO)_9$ and ($C_{12}(EO)_9$ + enzyme)

The CMC of the pure nonionic surfactant and in the presence of the enzyme were determined from surface tension measurements (KSV– Sigma 70 – Finland) at 4^{o}C.

Results and discussion

The kinetics of adsorption of alkaline phosphatase from its solution (0.30μg/mL) on LB films of DMPA-Zn^{2+} does not depend on the submersion time of the plates for the 0 to 30 minutes interval. All samples show an adsorbed mass of (241±10)ng. The presence of the enzyme was confirmed by the detection of the catalytic activity of the enzyme in the self-assembled film for the reaction of hydrolysis of p-nitrophenylphosphate (37°C, pH=9.4). Figure 1 shows the adsorption isotherm of the enzyme-surfactant complex to the LB films. It is possible observe in each curve a maximum, that changes with the surfactant concentration.

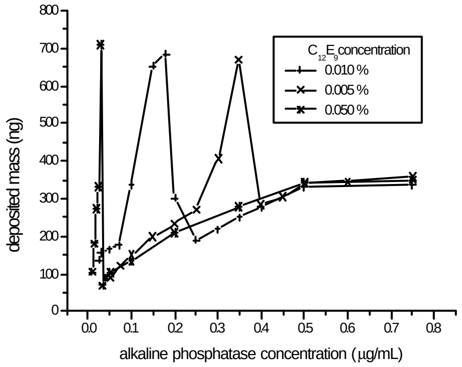

Figure 1: Adsorption isotherms (1°C, pH7.5) for AF/$C_{12}(EO)_9$ on DMPA-Zn^{2+} LB films, for three different stock solution concentrations of the non-ionic surfactant.

The concentration of the nonionic surfactant depicted in Figure 1 refers to the stock solution of alkaline phosphatase (7.5 μg/mL for the three curves). In this way, the $C_{12}(EO)_9$/enzyme ratio was maintained constant, once the solutions were further diluted with water. As the increase of alkaline phosphatase concentration was followed by the nonionic surfactant concentration it is possible to express the x-axis in terms of the surfactant concentration as well. In this way the maximum occurs for the three conditions of the Figure 1 at 2.2μg/mL of the nonionic surfactant.

From Figure 2 it is possible to observe that the enzyme-$C_{12}(EO)_9$ mixed system presents a lower CMC than the pure surfactant. The CMC in this case is $3.6x10^{-6}$ mol/L as compared to $7.1x10^{-5}$ mol/L for pure $C_{12}(EO)_9$. The first case correspond about 2.1μg/mL, that is approximately the polidocanol concentration at the maximum on the adsorption curve. Also, the adsorption of pure $C_{12}(EO)_9$ was carried out on LB films of DMPA/Zn deposited on QCM quartz crystals.

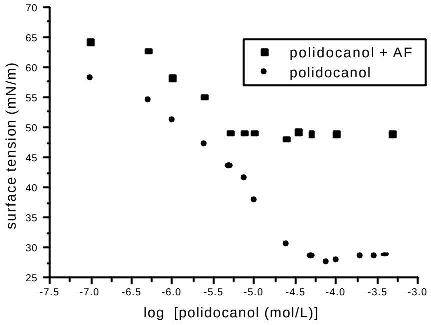

Figure 2: Surface tension curves (23°C) for: (●) pure $C_{12}(EO)_9$ and (■) $C_{12}(EO)_9$/ AF mixtures. The stock solution of AF was 7.5 μg/mL; AF concentration at the “break” is 0.18 μg/mL.

The maximum adsorbed amount is 70 ng, negligible in relation to the total mass of the enzyme. These data lead us to conclude that the phosphatase alkaline mass plays the major role in the adsorption of the mixed system

The results presented in this work show there are two regimes for the adsorption of the enzyme-surfactant mixed system on the phospholipid LB film. The first one, at a low concentration for both enzyme and surfactant, with the formation of multilayers. It occurs below the surfactant micelization. The second, above some critical concentration, that is related to the micelization for the mixed system. From Figure 1 one can observe that the three curves present a plateau at about 320 ng for the enzyme, for which there is a monolayer formation. The adsorption before the CMC is more abrupt because the enzyme is not yet in its solubilized form. So, the enzyme-enzyme interaction at the interface is favored when compared to the enzyme-surfactant interaction in solution.

Acknowledgements

This work was supported by FAPESP and CNPq/PADCT.

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BD-12 COLLOIDAL STRUCTURES AS COLORIMETRIC BIOSENSOR FOR AUTOANTIBODIES

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Introduction

Most of the previous applications of liposomes to immunodiagnosis have been based on homogeneous or heterogeneous immunoassays in solid phase. The conventional ELISA or liposome-enhanced ELISA [2] includes multi-steps for detection of antigen or antibodies. The diagnosis of autoimmune diseases is a complex issue, due to the heterogeneity of antibodies present in the sera of patients. Besides the heterogeneity of the autoantibodies, the multistep nature of this assay and the imprecise control of the density of antigens on the solid supports lead to a laboratorial variability of results obtained with different commercial kits, making diagnosis difficult [1].

Small polymerized unilamellar structures composed of polydiacetilenic acids and specific ligands on their surface may be useful for molecular recognition and signal transduction in the development of biosensors for diagnosis. These structures mimic cell surface molecular recognition as well as signal transduction in only one step.

This paper describes the characterization of polymerized structures containing cardiolipin as the affinity ligand for detection of antiphospholipid antibodies.

Experimental Methods

Cardiolipin (CL 98% pure) obtained by Sigma Chemical Co (USA) and 10,12-tricosadiynoic acid (TRDA) was synthetized in our laboratory. The colorimetric response, CR was calculated by eq. 1:

$$CR = [(B_0 - B)/ B_0] \times 100 \qquad (1)$$

where B_0 and B are the percentages of blue color before and after adding IgG solutions to liposomes. $B_0 = A_{blue} (A_{blue} + A_{pink}) \times 100$,; A_{blue} and A_{pink} are the absorbances on blue (635nm) and pink (480nm).

- **Preparation of polymerized structures**

The monomeric liposomes (1mM), composed of 20% cardiolipin and 80% 10,12-tricosadiynoic acid, were prepared by hydration of a thin film of their components. The structures formed were sonicated using a titanium probe instrument. The monomeric samples were polymerized in a 3mL quartz cuvette using a ultraviolet lamp (254 nm, 2.892mW/cm^2).

- **Characterization of monomeric and polymeric structures**

The monomeric and polymeric structures were characterized as to the mean hydrodinamic diameter for quasi elastic light scattering spectroscopy (He-Ne laser), at 90º. The photopolymerization reaction was monitored as a function of time by absorption spectroscopy and analysed by deconvolution of the spectra. The binding studies of autoantibodies were performed with specific immunoglobulin G (anti-cardiolipin) and non specific one. The results were expressed in terms of colorimetric response, CR.

Results and discussion

The mean diameter of fresh polymerized liposomes is similar to that of monomeric ones. And the mean diameter decreased during the first two months of storage, returned to its initial size after the third month and maintained this approximate size until the fifth month.

The chemometric treatment of the data from the spectra obtained, in the figure 1, permits a clearer observation of kinetics of liposome polymerization.

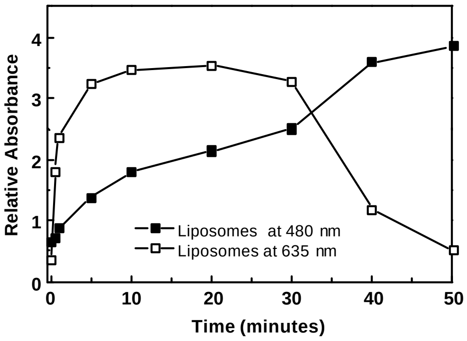

Figure 1 - Deconvolution of the spectra obtained during the polymerization of TRDA:CL (molar ratio of 80:20) liposomes.

In analyzing the curves, it could be observed that polymerization in the blue range remains approximately stable for between 5 and 20 minutes (635nm) while absorbance in the pink region increases (480nm). After 20 minutes the relative absorbance in the blue region decreases rapidly and the pink color of the solutions tends towards stabilization after 50 minutes. In 30 minutes, the purple color appears.

A comparison between the adsorption curves for specific IgG, IgG/anti-Cl, and nonspecific IgG by blue or purple polymerized liposomes is shown in Figure 2. The curve for data on purple liposomes is hyperbolic, whereas that for blue liposomes is a sigmoidal curve, indicating cooperativity between sites. At saturation, the IgG/liposome ratio was 1.74 10^{-12} U/liposome for purple liposomes and 4.06 10^{-12} U/liposome for blue ones.

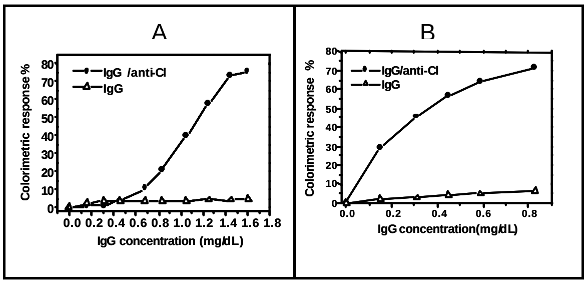

Figure 2 - Colorimetric detection of IgG for blue (A) and purple (B) polymerized liposomes (TRDA:CL molar ratio of 80:20). Concentration of the solutions: 0.5 mM total lipid.

Conclusions

The feasibility of polymerized liposomes for molecular recognition and transduction of the signal by a one-step colorimetric response was tested using specific and nonspecific anticardiolipin immunoglobulin G and cardiolipin as the affinity ligand. The colorimetric response was seven times higher for specific IgG compared with nonspecific ones, indicating the selectivity and specificity of the system.

This molecular recognition of autoantibodies and development of a one-step colorimetric signal represents a potentially important process for detection of autoantibodies and for the development of enhanced diagnostic kits.

Acknowledgements

FAPESP agency for the financial support and Debora Nakai for the help in chemometric analysis.

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BD-13 ADSORPTION OF IMMUNOGLOBULIN G FROM HUMAN SERUM USING PROTEIN L - AGAROSE

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Introduction

Several chromatographic methods have been proposed for the purification of biomolecules. However, the affinity adsorption is the most selective technique for purification of plasma proteins and removal of proteins from plasma in clinical apheresis [1].

Some biospecific ligands (protein A and protein G) have been used because they have a high selectivity towards the serum proteins. Other protein, named Protein L, which has a high binding affinity for the variable domain of human IgG kappa light chain has been isolated recently [2].

The present work focuses on the application of gel protein L-agarose for the purification of IgG κ light chains from human serum. The purification of IgG is generally required for the purposes of immunoaffinity chromatography and immunotherapy. Moreover, IgG removal is employed for the treatment of immune disorders, alloimmunization and cancer. For this eventual clinical applications gel protein L-agarose could be a potentially attractive tool, since some autoantibodies have IgG κ light chains.

Experimental

- **Materials**

Gel protein L-agarose was purchased from Sigma (St. Louis, MO, USA). Human Immunoglobulin G was purchased from Centeon Pharma GmbH (Marburg, Germany).

- **Methods**

Chromatographic procedures

Gel protein L-agarose was packed into a column (10 cm x 5mm I.D.) to give a bed volume of 1 mL. All chromatographic procedures were carried out at room temperature at a linear velocity of 0.5 mL/min with the ProTech Biochromatography System Gilson (Villiers-le-Bel, France).

For the experiments in dynamic mode (frontal analysis), purified IgG in 10 mM PBS buffer, pH 7.4 was pumped through the gel at concentrations ranging from 0.01 to 3.0 mg of IgG/mL until the available capacity of the adsorber was exhausted and the IgG concentration in the effluent approached the feed IgG concentration (the absorbance of the effluent reached a plateau).

Protein determination

Protein concentration in the serum was determined by the method of Bradford [3]. Crystalline bovine serum albumin was used as reference protein. Pure IgG concentration was determined by measuring the absorbance at 280 nm, with a molar absorptivity of 14 for a 1% of IgG.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The chromatographic fractions were analyzed in a SDS-PAGE (4-15% gradient gels) under non-reducing conditions using the Pharmacia Phast ™ system. The gels were silver-stained according to the method provided by the manufacturer.

Results and discussion

- **Influence of the buffer system on adsorption**

IgG adsorption onto the gel protein L-agarose seemed to depend on the buffer system used. Adsorption of purified IgG from human serum was studied at pH ranging from 7.0 to 8.0, but the optimal pH of adsorption was 7.0 for all the buffers (PBS, Hepes, Tris, Phosphate and Mops). Moreover, the Hepes allow a 1.2 to 2 times higher adsorption of pure IgG as compared to other buffers at pH 7.0.

- **Maximum binding capacity**

The maximum binding capacity of the gel protein L-agarose was determined in dynamic mode. The experimental data were analyzed using the Langmuir binding model, described by the equation:

$$Q_a = \frac{Q_x C}{K_D + C}$$

where K_D is the dissociation constant, C is IgG concentration at equilibrium, Q_a the amount of IgG adsorbed (determined after elution) and Q_x the maximum binding capacity of the gel. Figure 1 shows the IgG adsorption isotherm at 25°C.

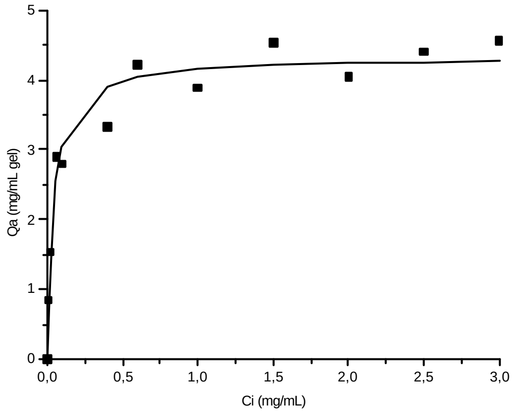

Fig.1: Adsorption isotherm of IgG in dynamic mode at 25°C.

The value of Q_x obtained by fitting the experimental results with the isotherm model using nonlinear least squares was 4.32 mg/mL gel. The value of K_D obtained was 2.8 x 10^{-7} M. This K_D of the complex IgG-protein L in the micromolar range indicates high affinity, which is typical for a bioespecific affinity ligand.

- **Adsorption studies of immunoglobulins from human serum**

To determine protein L-agarose selectivity towards the serum proteins, two kinds of equilibrium buffers were used: 10 mM PBS and 25 mM Hepes, both of them at pH 7.4. The amount of 0.5 mL of human serum 2 fold diluted in equilibrium buffer, was pumped through the column at a flow rate of 0.5 mL/min at room temperature. Desorption was performed by a step pH gradient (0.1 M glicine, pH 3.0 and pH 2.0).

SDS-PAGE under non-reducing conditions of the recovered protein fractions indicated that the retained proteins were mainly immunoglobulins and minor amounts of albumin and other serums proteins for both of the equilibrium buffers tested (results not shown).

Conclusion

This study has shown that IgG adsorption onto the gel protein L-agarose to depend on the buffer system employed.

The lower binding capacity of the gel protein L-agarose when compared to protein A-agarose is due to the fact that only IgG kappa light chains are adsorbed.

Acknowledgements

The authors would like to thank to CNPq (Brasília, Brazil) and Fapesp (São Paulo, Brazil), for the valuable financial support.

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BD-14 SINGLE STEP ISOLATION OF HUMAN COLOSTRUM IgA BY LECTIN AFFINITY CHROMATOGRAPHY

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Introduction

Lectins are proteins or glycoproteins of non immune origin; reversible and specific carbohydrate binding results in cellular agglutination [1]. In plants, these proteins can occur in vegetative tissues; leaves of *Bauhinia monandra* contain a galactose specific lectin, BmoLL, glutaraldehyde-treated rabbit erythrocyte active [2].

Biotechnological applications of pure lectins include their uses as affinity matrices to glycoconjugate fractionations; thus biologically active glycoproteins, such as immunoglobulins, can be isolated by chromatography on immobilized lectin for laboratory study or diagnosis of human diseases.

Polyvinyl alcohol (PVA) treated with glutaraldehyde (PVA-glutaraldehyde) constitute an interesting activated fibrous matrix for protein immobilization [3].

The aim of this work was to immobilize BmoLL in PVA-glutaraldehyde and evaluate BmoLL-PVA for colostrum glycoprotein isolation.

Experimental methods

- **BmoLL-PVA obtention**

BmoLL was incubated with PVA-glutaraldehyde under stirring overnight at 4°C. Following, the fibrous matrix was put in a column (1.0 x 5.0 cm) and 1.0 M

NaCl was added until no protein was detected in the collected fractions. The bound lectin was estimated by the difference between A280 nm before and after immobilization.

- **Colostrum preparation**

Human colostrum obtained from Hospital das Clínicas, Recife, Brazil, was clarified at 12,000 x g (20 min) and casein was precipitated at pH 4.6 with glacial acetic acid. Colostrum soluble proteins (CSP) were chromatographed on BmoLL-PVA.

- **CSP chromatography on BmoLL-PVA**

The chromatography was performed at 24°C; the column was washed with 0.1 M sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl, followed by application of CSP sample, proper buffer wash and 0.2 M galactose, 0.015 M sodium borate buffer, pH 7.4 or 1.0 M NaCl elutions. Fractions (1.5 ml) were collected at 15 ml/h and protein was monitored at A280 nm.

- **Electrophoresis**

Patterns of BmoLL-PVA desorbed proteins, CSP and human colostrum IgA (Sigma Chemical Company) were evaluated by SDS-PAGE carried out in 10% (w/v) gel.

Results and discussion

The coupling yield of BmoLL to PVA was 50%. The immobilization procedure did not abolish BmoLL activity nor induced steric hindrance since the matrix effectively retained proteins, bioselectively.

BmoLL-PVA chromatography was based on lectin-carbohydrate interaction [1]. Proteins were nonbioselectively eluted with borate and 1.0 M NaCl (Figure 1); these solutions were used to release glycoconjugates strongly bound to immobilized lectin [4].

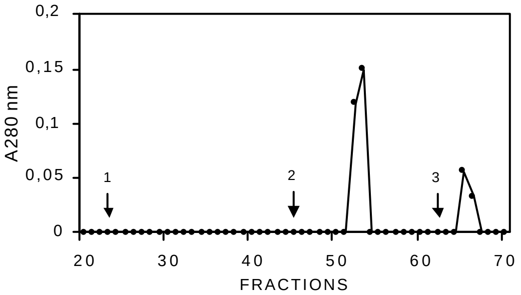

Figure 1. Chromatography of CSP on BmoLL-PVA. Desorbed proteins were detected at A280 nm. Arrows indicate the position were 0.2 M galactose (1), 0.015 M borate (2) and 1.0 M NaCl (3) were added to the column.

The purity of BmoLL-PVA desorbed proteins (borate or NaCl eluted fractions) by SDS-PAGE revealed a pattern similar to colostrum IgA. The chromatographic conditions did not alter lectin activity; the protocol was reproducible and demonstrated the stability of the matrix.

Conclusion

BmoLL was effectively immobilized in PVA-glutaraldehyde; the affinity matrix chromatography constitute a simple and inexpensive process for one step bioselective purification. IgA removed from human colostrum may serve as a valid reference protein for laboratory analysis.

Acknowledgements

This work was supported by CNPq.

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BD-15 FACTORS INFLUENCING THE ISOLATION OF PLASMA GLYCOPROTEINS BY *Cratylia mollis* LECTIN AFFINITY CHROMATOGRAPHY

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Introduction

Affinity chromatography is a type of liquid chromatography that makes use of biological interactions for separations and specific analysis of sample components [1]. The immobilized ligand is the key factor that determines the success of affinity chromatography method [2]. Thus, in contrast to other fractionation techniques such as gel permeation, reversed-phase and normal-phase chromatography, lectin column chromatography is of interest because the principle of its fractionation is based on the carbohydrate-biding specificity of the lectin [3,4], for example, lectins from *Canavalia ensiformis* (Concanavalin A or Con A) and from *Cratylia mollis* Mart (Cra) have both the ability to recognize and bind glucose/mannose residues [5]. Lectin affinity chromatography column have been used for separating and determining structure of glycoprotein-derived oligosaccharides and glycopeptides [4], and also for isolating plasma glycoproteins [6] and detecting glycoproteins with pathologically altered sugar chains [7]. Recently, serial lectin column chromatography has effectively been used for investigating the type of oligosaccharide structure, especially the N-linked oligosaccharides, within the glycoprotein molecule. Previous study from our laboratory showed that Cra lectin immobilized in an inert support (Sepharose) is able to isolate plasma glycoproteins [6]. In the present study, we evaluated whether the ability to isolate human plasma glycoproteins by Cra-Sepharose chromatography column is influenced by environmental condition (temperature).

Experimental methods

- **Plasma sample preparation**

Blood samples were collected from healthy subjects and anticoagulated with EDTA (1 mg/ mL). Plasma was separated by centrifugation (2,500 x g, 10 min) and dialyzed against phosphate buffer (20mM, pH 7.4). 4% (w/v) natrium phosphotungstate (1 mL) and 2M magnesium chloride (250 µL) were added to plasma sample (10 mL). The mixture was stirred for 30 min and centrifuged (30 min at 2,500 x g). The crude plasma preparation, free from low density lipoprotein and very low density lipoprotein, was dialyzed against 20mM phosphate buffer, pH 7.4, and used either fresh or stored at –20 ^{0}C. All the procedures were processed at 4^{o}C.

- **Preparation of Cra-Sepharose affinity chromatography column**

Cra lectin was purified, as described earlier [5], from extract of powdered *C. mollis* seeds in 0.15 M NaCl by protein precipitation with ammonium sulphate (fraction 40-60%) followed by affinity chromatography in Sephadex G-75. Pure Cra lectin (6.5 mg) was covalently attached to CNBr-activated Sepharose 4B (3 mL) according to the manufacturer's (Pharmacia) instructions. An affinity chromatography column (6.5 cm x 1.0 cm) was made with the Cra-Sepharose. Prior to use the column was equilibrated with 20 mM phosphate buffer, pH 7.4.

- **Isolation of Plasma glycoproteins**

Plasma samples (1 mL) were applied into the Cra-Sepharose column, and circulated through the column overnight, at a flow rate of 1 mL/h. After removal of the unbound proteins with approximately 60 mL phosphate buffer, the adsorbed material was eluted with various concentration of methyl α-D-mannoside/methyl α-D-glucoside (0.05-0.25 M). Fractions (1 mL each) were collected and the presence of proteins was estimated by mean of the absorbance at 280 nm. The bound fractions were dialyzed against several changes of distilled water, concentrated by freeze dry and quantified by Lowry's method [8].
The column was then regenerated with 20 mM phosphate buffer, pH 7.4, containing 0.5 M NaCl. The experiment was performed at 4^{o}C and at room temperature (26^{o}C).

Results and discussion

The concentration of plasma glycoproteins bound to Cra-Sepharose column at 26^{o}C was 44% lower than that bound at 4^{o}C. However, at 4°C the time of elution (120 min) was double (Figure 1) in comparison to that at room temperature (60 min).

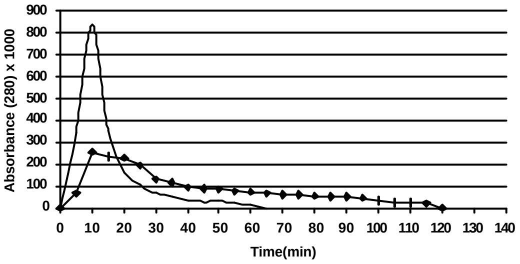

Figure 1. Elution of plasma proteins from the Cra-Sepharose chromatography column. Bound proteins were eluted with 0.5 M α-D-manoside/methyl α-D-glucoside, at 26 ^{0}C (—) and at 4 ^{0}C (–•–).

Furthermore, by using various concentrations of α-D-manoside/methyl α-D-glucoside the elution profile of plasma proteins bound to Cra column showed five peaks (Figure 2). The concentration of the elution solution was found to be of prime importance for separating the different peaks. Good separation was obtained over a large concentration range of methyl α-D-manoside/methyl α-D-glucoside (0.05-0.25 M).

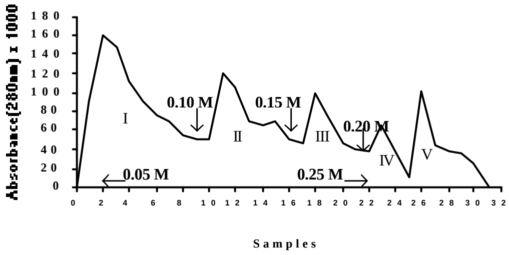

Figure 2. Elution of plasma proteins from the Cra-Sepharose chromatography column by using step-wise increases of α-D-manoside/methyl α-D-glucoside.

The interaction of oligosaccharide with lectin column is peripheral-structures dependent on each different outer-chain moiety [9]. Other lectins, such as Con A, have been used to identify the type of oligosaccharide chains. High mannose and hibrid oligosaccharides, which have many Man α1→and Man α1→2 residues, can bind more strongly to the columnn than complex type [10] under various concentrations of the elution solution. However, based only on the elution profile from an immobilized lectin column the structures of oligosaccharides in the glycoprotein can not be deduced. Finally, it should be considered that the elution pattern change with the chromatographic conditions. The temperature change the steric arrangement of the outer-chain moites, that interferes with the binding of oligosaccharides to the lectin column.

Conclusion

Our results suggest that isolation of plasma proteins by Cra-Sepharose affinity chromatography column depends on the temperature and concentration of the elution solution.

Acknowledgements

We are grateful to FACEPE, CNPq, BNB and CTPETRO for financial support.

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BD-16 IMMUNOLOGICAL CROSS-REACTIONS AMONG SEED PROTEINS OF *Cratylia mollis* AND OTHER LEGUMES

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Introduction

Seeds contain toxic proteins which participate of their defense mechanisms, such as protease inhibitors, enzymes and lectins, free or associated to storage proteins. There are evidences of structural relations among these proteins indicating a certain degree of molecular similarity, suggesting an ancestral common gene coding proteins with different activities inside the same plant.

Immunochemical reactions constitute efficient methods to evaluate structural relations among proteins. When antigenically similar proteins are detected there is a strong evidence of evolutionary and functional relations among them. Previous studies demonstrated that several plant lectins are immunologically related suggesting a highly conserved molecular region [1].

The availability in our Laboratory of pure proteins from *Cratylia mollis* seeds allowed the evaluation of the immunoreactivity among seed proteins from the above mentioned native forage and other species.

Experimental methods

- **Materials**

C. mollis seed proteins: isolectins (Iso) 1, 2, 3 and 4; Cratylin (a storage protein); CmtI (trypsin inhibitor); Lpase (leucine aminopeptidase) and Bpase (endopeptidase that hydrolyse benzoyl-arginine-p-nitroanilide).

Other proteins: lectins of *Canavalia ensiformis* (Con A) *Glycine max* (SBA), *Phaseolus vulgaris* (PHA) and *Ulex europaeus* (UEA I), *G. max* trypsin inhibitor (Bowman-Birk), adquired from Sigma Chemical Co. and Canavalin, a storage protein from *C. ensiformis*.

- **Methods**

Preparation of antibodies: antiserum against Iso 1, 2, 3 and Cratylin were developed by immunization of rabbits with purified proteins emulsified in complete Freund's adjuvant, the first time, and incomplete adjuvant, in subsequent doses, by intradermal injection of the purified proteins (200 μg).
Purification of IgG: IgG was purified in a protein A-Sepharose column (Amersham Pharmacia Biotech).

Double immunodiffusion tests: according to the method of Ouchterlony [2] and were performed using 1% (w/v) agarose gels in 0.15M NaCl. After incubation for 48 h in a humid chamber, at 4°C, the gels were washed several times in saline followed by destilled H_2O, and stained with Coomassie Blue.

Results and discussion

The results are showed in Tables 1, 2 and 3. Among *C. mollis* seed lectins, Iso 1 and 4, glucose/mannose specific, were immunologically related. There was no recognition between Iso 1 and Iso 2. Correia *et al*. (1996) [3], however, referred immunological reactivity between these two latter isolectins.

Data showed in Table 2 revealed immunoreactivity between *C. mollis* seed proteins. The storage protein, Cratylin, was not recognized by anti Iso 1 IgG and anti Iso 3 IgG. Anti Iso 2 IgG detected the storage protein; also, Cratylin anti IgG recognized Iso 2. Only anti Iso 3 IgG did not interact with *C. mollis* trypsin inhibitor or Bpase. Lpase, however, was strictly identified by anti Iso 1 IgG. The cross-reaction among proteins inside the same species was found to *Canavalia spp*. [4].

In relation to the immunological recognition among seed proteins from *C. mollis* and other legumes (Table 3), identity between Iso 1 and Con A was observed, according to data previously reported [3]. Anti Iso 2 IgG did not recognize Con A, even if both are glucose/mannose lectins which belong to the same subtribe (Diocleinae). In the same experiment it was also detected the reactivity between anti Iso 1 IgG and *G. max* trypsin inhibitor; the latter inhibitor was also recognized by anti Cratylin IgG.

Anti Iso 3 IgG behaved differently and did not recognize any other protein. This fact was not surprising since Iso 3 is a galactose specific glycoprotein, distinct from the other glucose/mannose isolectins.

C. mollis and *G. max* trypsin inhibitors were detected by anti Iso 1 IgG and anti Cratylin IgG. Anti Iso 2 IgG only recognized the inhibitor extracted from *C. mollis* seeds. These data suggest a certain degree of molecular homology between these proteins; they have different biological activities but similar physiological behavior in the plant, since both participate of defense mechanisms. Lectins and other defense proteins in *P. vulgaris* are evolutionary related [5].

Table 1
Cross-reactions among *C. mollis* seed lectins

IgG	LECTINS			
	Iso 1	Iso 2	Iso 3	Iso 4
Anti Iso 1	+	-	-	+
Anti Iso 2	-	+	-	-
Anti Iso 3	-	-	+	-

+ precipitation band observed
- no precipitation band observed

Table 2
Cross-reactions among *C. mollis* seed proteins

IgG	Proteins			
	Cratylin	Bpase	Lpase	CmtI
Anti Iso 1	-	+	+	+
Anti Iso 2	+	+	-	+
Anti Iso 3	-	-	-	-
Anti Cratylin	+	+	-	+

+ precipitation band observed
- no precipitation band observed

Table 3
Cross-reactions among proteins from *C. mollis* and other legumes

IgG Anti	Proteins					
	Lectins				Other proteins	
	Con A	PHA	UEA I	SBA	Can*	Bow**
Iso 1	+	-	-	-	-	+
Iso 2	-	-	-	-	-	-
Iso 3	-	-	-	-	-	-
Cratylin	-	-	-	-	-	+

+ precipitation band observed
- no precipitation band observed
***** Canavalin
** Bowman-Birk

Conclusion

The results revealed homologies among *C. mollis* seed proteins, even if they have distinct biological activities, indicating a common ancestral. Iso 1 and Con A are immunologically reactive. The highest cross-reactivity was obtained to Iso 1, suggesting the presence of an antigenic determinant, well conserved in evolution.

Acknowledgements

This work was supported by CNPq and CTPETRO.

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APGI SYMPOSIUM

MEMBRANE TRANSPORT

Tuesday, August 7, 2001

APGI Symposium Membrane Transport

8:30 a.m. ***Introduction***

8:40 a.m. **DETERMINATION OF THE PASSIVE ABSORPTION THROUGH THE RAT INTESTINE USING CHROMATOGRAPHIC INDICES AND MOLAR VOLUME**
Dr. Muriel Genty, Bristol-Myers Squibb, France

9:40 a.m. **MEMBRANE CROSSING, INTERNALIZATION AND INTRACELLULAR LOCALIZATION OF OLIGONUCLEOTIDES**
Dr. Trung Le Doan, CNRS UMR 8619, Institut de Biochimie Biophysique Moléculaire et Cellulaire, Université Paris Sud, France.

10:40 a.m. ***Coffee break and poster viewing***

11:30 a.m. **PARTICLE TRANSPORT ACROSS THE GI TRACT: A PATHWAY TO INDUCE ORAL VACCINATION ANDTOLERANCE**
Prof. Elias Fattal, School of Pharmacy, University of Paris Sud, France

12:30 – ***Lunch***

2.30 p.m. **NANOBIOTECHNOLOGY APPROACHES TO OVERCOME BIOLOGICAL BARRIERS TO DRUG DELIVERY AND TISSUE ENGINEERING**
Prof. Claus Michael Lehr, School of Pharmacy, Saarland University, Germany

3:30 p.m. ***Coffee break and poster viewing***

4:30 p.m. **INSTILLATION AND INTRAVITREAL INJECTION OF ANTISENSE OLIGONUCLEOTIDES IN THE EYE: COMPARISON BETWEEN SOLUTION AND LIPOSOMAL DOSAGE FORMS.**
Dr. Amélie Bochot, School of Pharmacy, University of Paris Sud, France, School of Pharmacy

5:30 p.m. **PHYSICO-CHEMICAL AND BIOLOGICAL EVALUATION OF THE NOVEL LONG CIRCULATING BIODEGRADABLE NANOCAPSULES AND THEIR APPLICATION ON THE PARASITIC DISEASES THERAPY.**
Dr. Vanessa Carla Mosqueira, Departamento de Farmácia - Escola de Farmácia, Universidade Federal de Ouro, Brazil.

CURRICULUM VITAE

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Education

1992: High School Graduation.

1998: Degree in Senior Engineering at the Ecole des Mines d'Albi, one of the French Grandes Ecoles.
Area of research: Process engineering, specialization in pharmaceutical processes.

1999: M.S. Degree in Pharmaceutical Technology and Biopharmacy at the School of Pharmacy of Châtenay-Malabry, France (Paris XI).
Area of research: Prediction of passive intestinal drug absorption using chromatographic capacity factors and physicochemical descriptors.

Since February 2000: Ph.D. student.
Area of research: Evaluation and comparison of various lipidic systems to improve the oral absorption of hydrophilic or lipophilic drugs.

Publication

M. Genty, G. González, C. Clere, V. Desangle-Gouty, J.-Y. Legendre, 2001. Determination of the passive absorption through the rat intestine using chromatographic indices and molar volume. European Journal of Pharmaceutical Sciences 12, 223-229.

PREDICTION OF THE PASSIVE ABSORPTION THROUGH THE RAT INTESTINE USING CHROMATOGRAPHIC INDICES AND MOLAR VOLUME

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Introduction

The ability to predict drug absorption through the gastro-intestinal barrier is a key issue in the selection of new drug candidates for oral delivery. However, the direct determination of permeability is not compatible with drug high throughput screening and alternative methods need to be developed. The use of artificial membranes to study the passive absorption of drugs has recently gained much interest. In particular, the chromatographic retention of solutes on immobilized artificial membranes (IAM) was shown to correlate with the drug lipophilicity and the extent of absorption for homologous series of drugs [1].

This study was aimed at evaluating the IAM chromatography, eventually coupled to the determination of simple physicochemical factors, as a predictive tool for the passive intestinal absorption of 12 structurally diverse drugs.

Experimental methods

- **Chemicals and their physicochemical characterization**

Acetylsalicylic acid and acetaminophen were purchased from Rhône-Poulenc (Courbevoie, France). Mannitol was supplied by Roquette (Lestrem, France). Niflumic acid was obtained from Hexachimie (Tonneins, france). Diazepam, sucrose, progesterone, testosterone, indomethacin, corticosterone salicylic acid, benzoic acid and polyethylene glycol 900 were supplied by Sigma (Saint Quentin Fallavier, France). ^{14}C benzoic acid, ^{3}H PEG900, ^{3}H corticosterone, ^{3}H progesterone, ^{14}C salicylic acid and ^{14}C indomethacin were purchased from New England Nuclear (Les Ulis, France). ^{3}H mannitol, ^{3}H acetaminophen and ^{14}C acetylsalicylic acid were obtained from Isotopchim (Ganagobie Peyruis, France). ^{14}C sucrose, ^{3}H cyclosporine, ^{14}C diazepam and ^{14}C testosterone were supplied by Amersham (Orsay, france).

The physicochemical descriptors which influence passive intestinal absorption [2], i.e. molecular weight (M_w), molar volume (V_x), solubility at saturation in water (C_s), pKa, melting point T_M, octanol/water partition coefficient (log $P_{o/w}$) and hydrogen bonding (δ_h), were obtained for the 12 molecules under investigation.

- **Immobilized Artificial Membrane Chromatography**

The chromatographic retention time of each compound was determined on a IAM PC DD 2 column (Regis Technology, Morton Grove, IL, USA) according to a protocol described previously [3]. Chromatographic retention indexes are expressed by the logarithm of capacity factor, logk'$_{IAM}$, calculated as follows :

$$\log k'_{IAM} = \log\left(\frac{t_r - t_0}{t_0}\right)$$

where t_r is the retention time of the drug and t_0 the hold-up time of the column.

When a compound was highly retained on the column so that t_r could not be measured accurately in 100% aqueous phase, its logk'$_{IAM}$ was obtained from extrapolation to the x coordinate of the linear plot of logk'$_{IAM}$ versus the fraction of acetonitrile.

- **In vitro absorption through rat gut sacs**

2.5cm length gut sacs were prepared from the everted intestine of Male Sprague Dawley rats. Each sac was placed in a solution of the studied marked molecule in culture medium. After incubation, the sacs were removed and samples of the external medium, serosal fluid and of the tissue digest were assayed for radioactivity. The protein content of the digest was determined spectrophotometrically with bovine serum albumin as the standard.

The transport of the molecules into the tissue and from the mucosal to the serosal side of the mucosa was calculated as nmol transported per mg of sac protein per 30 minutes. In the particular case of molecules presenting a carrier-mediated transport system, sacs were not everted to obtain their passive intestinal absorption.

- **Correlation analyses**

Stepwise Multiple linear Regression was used to select among a set of descriptors those which correlated best with the dependent variable. As the relationship between physico-chemical descriptors and P_a may be non-linear Back Propagation Neural Network analysis (BPNN) was also used [4].

Results and discussion

MLR did not show any significant correlation between P_a and a single descriptor of either the molecular size (M_x, V_x), the crystalline cohesion T_M

or the solvatation by hydrogen bonds (pK_a, C_s, δ_h). $P_{o/w}$ correlated to some extent with Pa, albeit with a poor correlation coefficient (r=0,70). MLR showed that the factor which correlated best with passive intestinal permeability was the IAM capacity factor $\log k'_{IAM}$ (r=0.88 for the set of 12 molecules).

In order to further improve the model, the combination of another physicochemical descriptor with $\log k'_{IAM}$ was investigated. MLR showed that the prediction of the permeability for the 12 molecules was improved when the molar volume was added to $\log k'_{IAM}$ (r=0.91) (Figure 1).

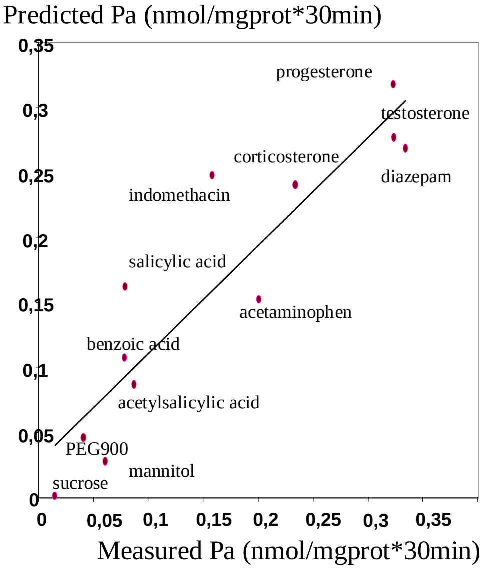

Figure 1 Correlation between measured P_a and predicted P_a by Multiple Linear Regression analysis with $\log k'_{IAM}$ and V_x as descriptors.

The improvement of the prediction of permeability when V_x is added to $\log k'_{IAM}$ as a descriptor may be explained by the fact that the permeation of a drug through a membrane not only depends on the equilibrium membrane partition coefficient, but also on the membrane diffusion coefficient of the solute and on the membrane thickness. Indeed, permeation Pm of a drug through a membrane is expressed as :

$$Pm = \frac{DmKm}{L}$$

where Km is the equilibrium membrane partition coefficient, Dm the membrane diffusion coefficient of the solute and L the membrane thickness.

Despite non-specific interactions between drugs and IAM, we and other have shown that $\log k'_{IAM}$ correlates well with the ionization-corrected partition coefficient [3]. Therefore, k'_{IAM} does reflect K_m. The second descriptor V_x may correctly model drug diffusion Dm in the membrane. Indeed, D_m primarily depends on the molecular size and decreases as molecular size increases.

Owing to its ability to model non-linear relationships, BPNN was also applied to the data collected in this study. Like MLR, BPNN showed a highly significant prediction of the passive intestinal permeability when V_x and $\log k'_{IAM}$ were used as input nodes (r=0.94) (Table 1), demonstrating that BPNN is particularly relevant for the study of such correlations [4].

Table 1 Passive permeability values of the 12 molecules under investigation measured experimentally or calculated using BPNN analysis

Compound	Measured Pa[a] (nmol/mgprot*30min)	BPNN calculated Pa (nmol/mgprot*30min)
Sucrose	0.015 ± 0.001	0.035
PEG900	0.041 ± 0.011	0.021
Mannitol	0.061 ± 0.011	0.056
Benzoic acid	0.078 ± 0.007	0.121
Salicylic acid	0.079 ± 0.025	0.143
Acetylsalicylic acid	0.087 ± 0.015	0.103
Indomethacin	0.158 ± 0.025	0.241
Acetaminophen	0.201 ± 0.024	0.138
Corticosterone	0.234 ± 0.028	0.186
Progesterone	0.323 ± 0.051	0.333
Testosterone	0.324 ± 0.049	0.318
Diazepam	0.334 ± 0.032	0.298

[a] Results are the mean ±S.D. of 21 sacs for mannitol, 3 to 6 sacs for the other compounds.

Conclusion

This study demonstrates that retention indices on IAM and calculated molar volumes can be used concurrently to predict the intestinal passive absorption of structurally diverse drugs. Both terms can easily be integrated into high throughput screening.

Acknowledgements

The excellent assistance of Didier Roy, Anne Laumain and Stéphane Desvignes is gratefully acknowledged.

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CURRICULUM VITAE

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1960-1963: Graduate (Ingénieur Chimiste) from the Ecole Supérieure de Chimie Industrielle de Lyon
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Scientific Activities :

Research: Our actual research topics cover the domains of Chemistry and Cellular Pharmacology of Nucleic Acids (oligonucleotides and genes). Current research dealts with the study of binding, internalization and intracellular localization of oligonucleotides in vascular smooth muscle cells by different methods: fluorescence microscopy, cell fractionation and ionic microscopy. This basic research serves as background for the comprehension of the antiproliferative effects of antisense oligonucleotides during the restenosis process. Other aspects of our research involve the intra-arterial delivery of oligonucleotides incorporated into a polymer-coated stent.

Publications: 66 papers

Patents: 2

Encapsulation of oligonucleotides in polycyanoacrylate nanoparticles for in vivo delivery, French Patent* N° 89/09209.
Coated stents for in situ delivery of oligonucleotides and other bioactive agents, Patent N° WO 99/03517.

Teaching : Université Paris Sud – Orsay :

- Master of Science Degree in Biology.
  Subject: "Technologie des macromolecules informatives"
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  "Chemistry and Biology of Nucleic Acids".
- European summer school "First Intensive Socrates Programme on Innovatives Therapeutics, from molecules to drugs.

MEMBRANE CROSSING, INTERNALIZATION AND INTRACELLULAR LOCALIZATION OF OLIGONUCLEOTIDES

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Abstract

The antisense concept is simple but its application to cellular systems appeared more complex. Uptake of oligonucleotides either as such or combined with transfection reagents, by cells proceed via different routes including receptor-mediated endocytosis, pinocytosis and other pathways that have recently been described (oligonucleotide channel, potocytosis, caveolea-mediated process). Cell internalization and subcellular distribution depend on the type of cell, the chemical structure of the oligomer and several other parameters that will be discussed. The way oligonucleotides route inside the cell will determine their chance of surviving lysosomal degradation, their meeting target macromolecules and the incidence of their side effects or cytotoxicity. Knowledge of factors governing the binding, internalization and trafficking of oligonucleotides could be of great help in the rational design of antisense agents. Cell penetration may not be a crucial problem for antisense applications as ODN can be found in high amount inside many cell types. The real problem rests on the bioavailability of the internalized compound in the right compartments i.e. the cytosol and the nucleus. Bioavailability in this context means sufficient amount and ability to bind the RNA and DNA targets instead of cytosolic proteins. For those cells where normal endocytosis occurs in parallel to other processes that do not involve vesicular transport of ODN, there may be a favourable partition of the antisense molecule in the cytosol and the nucleus and thereby a higher chance to achieve an antisense reaction. This presentation aims to describe some of the most recent advances in the study of cellular interactions of oligonucleotides. The presented data could be useful for those who intend to use antisense molecules as tools for fundamental cell biology studies (specific knockdown of protein) or as potential therapeutic agents in the treatment of various diseases (oligonucleotide-based therapeutics).

Acknowledgements

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CURRICULUM VITAE

Elias Fattal

Elias Fattal is a full professor of Pharmaceutical Technology at the University of Paris-XI in Châtenay-Malabry, France. He received his Pharmacy Degree (1983), and Ph.D. (1990) from the University of Paris-XI. After visiting the Department of Pharmaceutical Chemistry, University of California, San Francisco for a post-doctoral position (1990-1991), he became associate Professor (1992) and full Professor at the University of Paris-XI (2000). His research activity deals with the design of polymeric nano and microparticles and liposomes for the delivery of peptides/proteins and nucleic acids. In 1999, he received the Colloidal Drug Carrier Award (at the 5th Expert meeting on colloidal drug carriers, Berlin, Germany). He is the author and co-author of 82 publications and book chapters and 3 patents. Elias Fattal is the co-editor of STP Pharma Sciences, Vice President of APGI and member of the board of the GTRV (French Scientific Group on Drug Targeting). He is also the coordinator of a European Socrates Intensive Program named « Innovative therapeutics : from molecules to drug ».

PARTICLE TRANSPORT ACROSS THE GI TRACT: A PATHWAY TO INDUCE ORAL VACCINATION AND TOLERANCE

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Introduction

Mucosal admistration of antigens by mucosal routes is able either to induce a mucosal immune response or a suppression of the systemic immune response called tolerance. However, antigens are generally degraded by proteolytic enzymes before reaching the priming site (e.g. Peyers patches in the gut). Nevertheless, encapsulation of vaccines into biodegradable microspheres provides excellent mucosal immunogens with a high potential for immunization or the induction of oral tolerance since it allows both protection against degradation and targeting of the follicle sites. We present here the possibilities of using poly(lactide-co-glycolide) microparticles to design an antibacterial vaccine and to induce oral tolerance against a milk protein.

Mucosal delivery of an antibacterial antigen

Phosphorylcholine (PC) was chosen as an antigen because it was found to elicit an immune response after intestinal exposure of mice to PC-bearing Salmonella typhimurium, and because anti-PC immunity protects mice against Streptococcus pneumoniae, another PC-bearing micro-organism [1]. This hapten is also present on different microorganisms that invade or colonize different mucosa [1].

A PC-Thyroglobulin (PC-Thyr) conjugate was encapsulated in Poly (DL-Lactide-co-glycolide) (PLG) prepared by the multiple emulsion solvent evaporation method. Particles of a size between 1 to 10 µm were obtained displaying a high encapsulation efficiency and a low burst release in simulated gastric fluids [2]. Morphological characterization of the microparticles suggests that some of the protein is located on the surface tightly bound to polymer. However, this strong attachment to the polymer did not modify the antigenicity of the conjugate as shown by ELISA measurements [2].

PC-Thyr was followed by a specific antiphosphorylcholine systemic, intestinal, pulmonary and vaginal immunity [3]. These first results suggested that the potentialities of PC-Thyr loaded microspheres to protect against infections caused by PC-bearing microorganisms which invade or colonize at different sites should be further explored. Therefore, we tested the protective immunity elicited by intragastric vaccination with PC-Thyr encapsulated in PLG microspheres against *Salmonella typhimurium* in a mouse model of invasive intestinal infection. Mice were primed intragastrically on days 1, 2 and 3 and boosted on days 28, 29 and 30 with PC-Thyr (280 µg) encapsulated in DL-PLG microspheres, free PC-Thyr, or blank microspheres. A significant rise in anti-PC IgA titers, as measured by ELISA, was observed in the intestinal secretions after immunization with PC-loaded microspheres, compared to the titers of mice immunized with free PC-thyr or blank microspheres [3]. This antibody response peaked 14 days after the last boost and correlated with a highly significant resistance to oral challenge by *S. typhimurium* strain C5 (fig.1) [3]. Control mice were primed intraperitoneally on day 1 with 15 µg PC in complete Freund's adjuvant, and boosted on days 10, 14 and 20, with the same dose without adjuvant but via the same route. In these mice, the levels of anti-PC IgA in intestinal secretions were equivalent to those of the mice intragastrically immunized with PC-loaded microspheres, but protection was significantly weaker, suggesting that either the IgAs were not functional or that other immune mechanisms are important in protection [3].

It was also shown that both free PC-Thyr and PC-Thyr loaded microspheres administered intranasally also confer a strong mucosal and systemic anti-PC immune response. This immunity was protective against an intranasal lethal challenge by *S. pneumoniae* serotype 3, while protection following oral immunization with the same preparations was significantly lower [4]. These results suggest that PC vaccination, yet effective against *S. typhimurium C5*, may be a promising approach for the development of a combined mucosal vaccine. However, site-targeted immunization may be crucial for promoting effective local immune responses in the desired epithelial tissues.

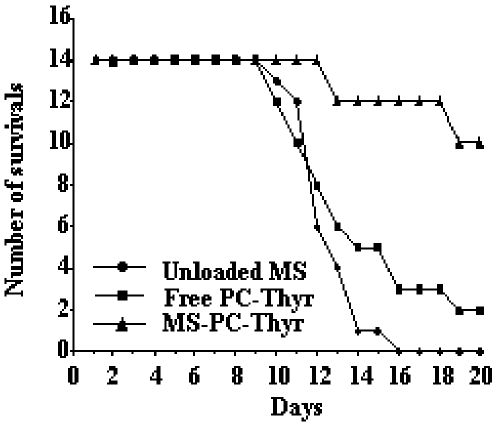

Figure 1: Mice survival after immunisation with PC-Thyr containing microspheres

Mucosal delivery of a dietary antigen

Allergies to milk proteins are frequently encountered in the new born population. In order to prevent this allergy by inducing oral tolerance, one of the major allergenic milk protein, β lactoglobulin (BLG) was entrapped into biodegradable Poly(lactide-co-glycolide) microspheres prepared by the multiple emulsion solvent evaporation method and was then orally given to mice.

The goal of the formulation study was to associate large amounts of proteins to the smallest amount of polymer so that a minimal quantity of microspheres would be administered. It was shown that introducing tween 20 in the formulation was able to increase the encapsulation efficiency and to better control protein release reducing the burst release effect [5]. Moreover, Oral administration of microspheres containing BLG reduced significantly (by 10, 000) the amount of protein necessary to decrease both specific anti BLG IgE and DTH response. In conclusion, microspheres appear to be optimal systems to induce oral tolerance [6].

Conclusion

Taken together, our results highlight the potential of antigen encapsulation in DL-PLG microspheres for eliciting protective immunity against invasive intestinal bacterial diseases and the induction of oral tolerance against a dietary antigen.

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CURRICULUM VITAE

Claus Michael Lehr

Professor Lehr studied pharmacy at the Universities of Mainz and of Hamburg, and received his German "Approbation" in 1987. In 1991, he received his Ph.D. from Leiden University, The Netherlands. After a postdoctoral fellowship (1991-1992) at the University of Southern California, Los Angeles, he worked as a research scientist at the Leiden Amsterdam Center of Drug Research. In 1993, he was appointed as Associate Professor at Philipps-University Marburg, Germany, in 1993. In 1995, he moved to Saarbrücken, Germany, to become Full Professor and Head of the Department of Biopharmaceutics and Pharmaceutical Technology at Saarland University. In 1998, he co-founded ACROSS BARRIERS GmbH, a company dedicated to new technologies for drug absorption and delivery.

Prof. Lehr’s scientific interest is the controlled delivery of macromolecular drugs (peptides, proteins, gene vectors) across biological absorption barriers, in particular to the mucosal epithelia of the gastrointestinal and respiratory tract. For such purposes, the research at his department is concerned with bioadhesion technologies (mucoadhesive polymers, cytoadhesive lectins), colloidal drug carriers (liposomes and nanoparticles), as well as with epithelial cell culture systems, non-viral gene delivery systems an tissue engineering.

Professor Lehr is a member of the Controlled Release Society (CRS), the American Association of Pharmaceutical Scientists (AAPS, the German Pharmaceutical Society (DPhG), and the European Federation of Pharmaceutical Societies (EUFEPS). He is European Editor of the European Journal of Pharmaceutics and Biopharmaceutics, and he serves as a member of the editorial boards of the Journal of Drug Targeting, the European Journal of Pharmaceutical Sciences and PharmSci. He is a member of the Executive Committee of APV, a scientific society dedicated to the applied Pharmaceutical Sciences and Technology in Germany and in Europe. In august 1999 he was also elected into the council of EUFEPS, and in 2000 he won the Young Investigator Award of the international Controlled Release Society.

NANOBIOTECHNOLOGY APPROACHES TO OVERCOME BIOLOGICAL BARRIERS TO DRUG DELIVERY AND TISSUE ENGINEERING

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INTRODUCTION

Pharmaceutical nano-biotechnology has an enormous to improve the safety and efficacy in delivering drugs across biological barriers, both for local as well as for systemic application. This holds in particular for for macromolecular biopharmaceuticals (peptides, proteins, antisense agents, gene vectors), but also for conventional small organic molecules.

In our laboratories, we are exploring the potential of nanoparticulate carriers and nanostructured surfaces for drug delivery and tissue engineering relevant to epithelial barriers. This may lead to improved drug delivery systems, in-vitro test systems or medical products, such as artificial vascular grafts.

Our approach comprises the following steps:

- Development of **new platform technologies** for the preparation of nanometer-sized drug carriers;
- Identification and optimization of **bio-specific ligands for surface modification** to control cellular binding, uptake and transport;
- Development and characterization of **epithelial cell culture systems** relevant to drug delivery and tissue engineering

METHODS

Cationically surface modified silica nanoparticles as DNA-carriers

Cationic silica nanospheres (SiNP) were obtained by covalent modification of commercial SiO_2 -colloids with aminosilanes [1,2]. The size could be varied between 10 and 100 nm and a positive zeta potential of more than +25 mV at pH 7.4 could be obtained.

Lectin-mediated recognition of cell-bound glycoproteins

Lectins are proteins of non-immunological origin with the capacity to specifically recognize oligosacharides. Their potential as second-generation bioadhesives to control cellular binding and transport has been discussed before[3]. In this study, we have used computer-aided modelling and docking, as well as atomic force microscopy to calculate and measure binding forces, respectively, relevant to the design of structulrally optimized lectinomimetics for controlled cellular targeting and delivery.

Biodegradable PLGA-Nanoparticles

The anti-inflammatory drug rolipram was nano-encapsulated in poly[lactic-co-glycolic acid] (PLGA) by an optimized oil-in-water emulsion technique [4] in order to demonstrate the potential of such nanoparticles for the targeted delivery to ulcerated areas of the colonic mucosa in a relevant animal model in-vivo.

Surface modification of vascular grafts

Commercially available PTFE-grafts were first oxidized to produce free hydroxyl groups. Cyanuric chloride served as a cross-linker between the OH groups and the amino functions of cell adhesion molecules derived from a bacterial invasion factors.

RESULTS AND DISCUSSION

DNA-binding, Transfection-efficacy and cytotoxicity of SiO_2–nanoparticles

SiO_2 is a relatively inert and well tolerated biomaterial. Although it is very common as a filler and lubricant in tablets and other conventional oral dosage forms, its use for the develoment of advanced drug carriers appears to be relatively unexplored. As a result of our studies, we found that Cationically surface modified silica nanoparticles forme stable complexes with DNA under physiological condidtions which were able to transfect epithelial cells in vitro with marker genes. Transfection rates were comparable to those observed with other non-viral gene tranfer systems (e.g. pEI and other polymers.) Surprisingly, the cytotoxicity of the Silica-nanoparticles was much better than for other systems, Such favourable safety profile underscores the potential of this new technology platform especially for the oral route of application.

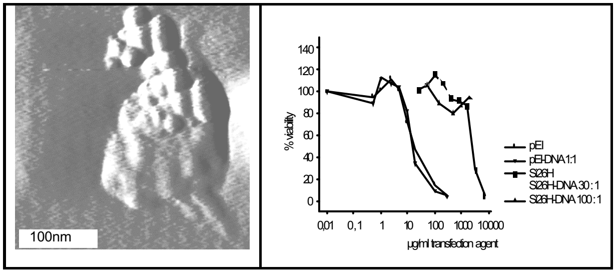

Figure 1: left: Atomic force micrograph of DNA-SiO_2 –„nanoplex“, **right**: favourable toxicity profile of SiNP compared to polymeric transfection agent pEI

Prediction and evaluation of lectin-mediated bioadhesion by computational docking and atomic force microscopy

The binding energy of the bioadhesive wheat germ agglutinin (WGA) to various oligosaccharides was calculated in-computro. These values showed an excellent correlation with the experimentally determined $IC_{50\%}$-values of these sugars to inhibit bioadhesion of WGA to Caco-2 monolayers in-vitro (Fig. 2a).

Atomic force microscopy (AFM) using lectin-modified cantilevers on phospholipid monolayers allowed to localize and quantify binding to glycosylated domains by point spectroscopic force measurements (Fig. 2 b,)

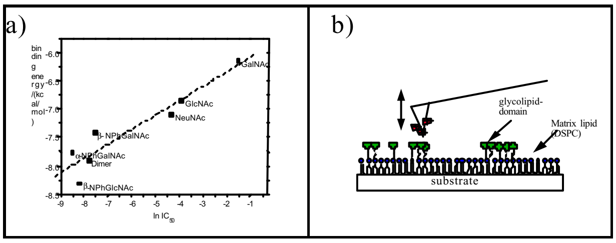

Figure 2a) Correlation of calculated vs experimentally determined lectin binding; **b,)** verification of bioadhesion by lectin-modified AFM probe

Site-specific delivery of nanoparticles to inflamed colonic mucosa

After demonstrating size dependent bioadhesion of nanoparticles to the inflamed colonic mucosa[5], biodegradable PLGA-nanoparticles containing the antiinflammatory drug rolipram were administered to TNBS-induced colitic rats. Compared to the same dose of the free drug (administered as solution), nanoparticle formulations lead to a prolonged amelioration of clinical effect scores and a reduction of adverse neurotropic effects.

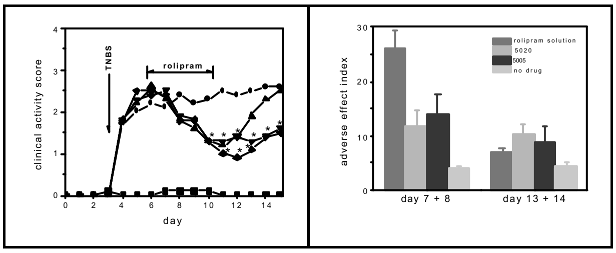

Fig. 1: Clinical activity score (left) and adverse neurotropic effects of rolipram nanoparticles in-vivo (TNBS-colitic rats); Legend: ■ = healthy control; ● = colitis control; ▲ = rolipram solution; ▼ = NP-5020; ◆ = NP-5005.

Endothelialization of vascular grafts

The modification of inert PTFE grafts with biologically active molecules allowed confluent growth of human endothelial cells, withstanding the shear stress encountered under physiological perfusion conditions (Fig 3). Potential applications include prosthetic bypasses in cardiovascular surgery,.

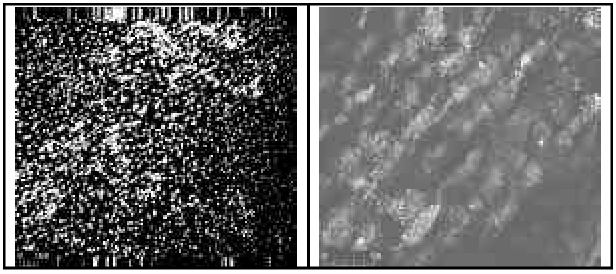

Figure 3, left: Adhesion of human endothelial cells to invasin-modified surface leads to almost complete coverage; **right**: polymer-grown endothelial cells differentiate as detected by expression of specific marker proteins.

CONCLUSIONS

Pharmaceutical nano-biotechnology, i.e. the design of nanoparticulate carriers and nano-structured biomaterial surfaces, promises an excellent potential for both drug/gene delivery and tissue engineering..

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CURRICULUM VITAE

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Amélie Bochot was born in 1969. She received her Pharmaceutical Degree in 1993 (University of Dijon, France) and her Ph.D. in Pharmaceutical Technology in 1998 from the University Paris XI, France. (Research advisors: Pr. Patrick COUVREUR and Dr. Elias FATTAL). The subject of her thesis was the design of a new eye delivery system for oligonucleotide based on a liposomal dispersion in a poloxamer gel. In 1998, she joined the group headed by Pr. D. Duchêne and G. Ponchel (UMR CNRS 8612, University of Paris XI) as an Associate Professor in Pharmaceutical Technology. In 1999, she received an award for her thesis (Prix de la Paul Neumann Foundation) and is member of the APGI board. In 2001, she joined Elias Fattal's department research focused on the targeting of fragile molecules using dispersed systems (liposomes, microspheres, nanoparticles, emulsions). Her main research interests are the conception and the evaluation of new topical drug delivery systems using liposomes, hydrogels and/or cyclodextrins.

INSTILLATION AND INTRAVITREAL INJECTION OF ANTISENSE OLIGONUCLEOTIDES IN THE EYE: COMPARISON BETWEEN SOLUTION AND LIPOSOMAL DOSAGE FORMS.

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Introduction

Antisense oligonucleotides with base sequences complementary to specific genetic targets offer the possibility of selectively modulating the expression of gene. It has been shown that antisense oligonucleotides possess significant inhibitory activity against a number of DNA viruses responsible for ocular diseases: herpes simplex virus (HSV1 and HSV2) in the anterior segment of the eye, human cytomegalovirus (CMV) in the posterior segment of the eye. This strategy has recently led to the first antisense oligonucleotide used in therapeutics. Vitravene® (Fomivirsen) is a 21-mer phosphorothioate oligonucleotide injected intravitreally once weekly during induction and every 2 or 4 weeks during maintenance for the local treatment of cytomegalovirus (CMV) retinitis in patients with acquired immunodeficiency syndrome (AIDS). Unfortunately, the use of these molecules is still limited by their poor biological stability in biological fluids.

Only a small portion of ODN reaches the internal eye tissues after instillation due to the protective functions of the eye against the entry of foreign compounds and the high hydrophilicity of ODN. One strategy to improve the protection of oligonucleotides from degradation consists in their encapsulation within liposomes which, in addition, present further advantages as ocular delivery systems: they can provide increased efficacy, reduced toxicity as well as prolonged activity. Concerning the treatment of posterior segment disorders, the efficacy of conventional formulations for drug administration is also limited while direct injection into the vitreous humor is very delicate. Furthermore, repeated injections are required by the short intraocular half-life of drugs when administered intravitreally. To improve the condition of intravitreal delivery, liposome dosage forms can be considered as interesting systems [1].

The main goal of this study was to evaluate the efficacy of liposomes and liposomes dispersed within a thermosensitive gel for delivering a model oligonucleotide after instillation and intravitreal injection, through a slow release process compare to a control solution. Poloxamer 407 is a copolymer of polyoxyethylene and polyoxypropylene which offers the unique property of reversible thermal gelation. This allows instillation or injection in the eye of a fluid solution, which forms a semi solid gel at physiological temperature.

Material and Methods

- **Materials**

Soybean Phospholipid (PC) was provided by Lipoid (KG, Ludwishafen, Germany). 1, 2-distearoyl-sn-glycero-3 Phosphatidylethanolamine -N-[Poly(ethyleneglycol)-2000] (PEG-DSPE) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). Cholesterol (CHOL) was obtained from Sigma Chemical Co (St Louis, MO, USA) and the model oligonucleotide 5'-phosphorylated oligothymidylate (pdT16) from Eurogentec (Belgium). Poloxamer 407 was a gift from BASF.

- **Methods**

Liposome preparation

PC:CHOL:DSPE-PEG2000 (molar ratio 64/30/06) liposomes were produced by the method described by Bangham [1]. To calibrate the liposome diameter the suspensions were extruded, using an extrusion device, through polycarbonate membranes with diameters of 0.4µm and 0.2µm, respectively. A radiolabelled model oligonucleotide, ^{33}pdT16, was encapsulated in the liposomes by the freeze/thaw technique [2]. Free pdT16 was removed by ultracentrifugation of the vesicles.

Liposomes dispersed within poloxamer gel

Poloxamer 407 gel was prepared by the cold process described by Schmolka [3]. Liposomes were dispersed within poloxamer 407 gels under stirring at +4°C to get a final poloxamer concentration of 27% (w/v).

- ***In Vivo studies***

Instillation

The distribution of pdT16 in several ocular tissues (cornea, conjunctiva, sclera, iris, lens, aqueous and vitreous humors) was determined in the rabbit after instillation of various dosage forms. Radiolabelled pdT16 was applied as a simple solution, a 27% poloxamer 407 gel, a suspension of liposomes or liposomes dispersed within a 27% poloxamer 407 gel. pdT16 concentrations were measured in the tissues and fluids by radioactivity counting at time intervals of 10 minutes, 2 hours and 24 hours.

Intravitreal injection

Ocular distribution and clearance from the vitreous humor of pdT16 were evaluated during 30 days in the rabbit by radioactivity measurements after intravitreal injection of either a solution or liposomes containing pdT16 oligonucleotide or liposomes dispersed within poloxamer gel. The integrity of pdT16 was investigated using a competitive hybridization assay.

Results and Discussion

In a first step, the distribution of pdT16 after instillation was performed. When the pdT16 solution was used, the highest concentrations were observed in the conjunctiva and the cornea, while a substantial amount of drug was also present in the sclera. Low concentrations were measured in the iris. Using the same treatment protocol, the two liposomal formulations (liposomes dispersed in buffer or liposomes dispersed within the poloxamer gel) delivered low drug amounts of pdT16 to all ocular tissues, and particularly to the conjunctiva and the cornea. The poloxamer gel provided higher tissue concentrations of pdT16 than liposomes but lower than those observed with the solution. These findings indicated that liposomal forms may not be considered useful delivery systems for topical administration of oligonucleotides in superficial ocular diseases [3].

The residual concentration of pdT16 oligonucleotide within the ocular tissues was significantly increased after intravitreal administration of the liposomal suspension instead of a single solution. 14 days postinjection, pdT16 within the vitreous humor using the liposomal suspension in buffer, was 9.3-fold higher than that obtained with pdT16 in solution and 4-fold higher than that measured with the liposomes dispersed within the gel. In the vitreous humor, pdT16 administered with liposomes dispersed within poloxamer gel was cleared faster than pdT16 injected within a simple liposomal suspension, although this difference was only visible after 1 day. The shorter pdT16 residence time within the vitreous obtained with the liposomes-gel was probably due to a faster pdT16 release from the vesicles induced by the presence of the poloxamer 407 forming the gel. Indeed, we have previously demonstrated that poloxamer 407, exhibited a different behavior with respect to liposomes depending on its concentration in the formulations [4]. In dilute solution, poloxamer 407 exhibited surfactant properties able to destabilize the liposomes, which resulted in a considerable leakage of encapsulated pdT16 whereas the same liposomes dispersed within a high poloxamer concentration (27%) were stable. Moreover, we have observed that 27% poloxamer gels could be dissolved in aqueous media. In a consequence, the gel state disappeared to form a dilute poloxamer solution

Administration of liposome-encapsulated pdT16 oligonucleotide resulted in a sustained release into the vitreous and the retina-choroid comparatively to the solution and in a reduced distribution in non-relevant tissues (sclera, lens). In addition, liposomes have allowed the protection of the phosphodiester oligonucleotide against degradation. This was not obtained after administration of the oligonucleotide free.

Conclusion

In conclusion, liposomal dosage forms may not be considered useful delivery systems for instillation of oligonucleotides in superficial ocular diseases.
However, the data obtained in this work show for the first time the beneficial effect of liposomes for the intravitreal injection of phosphodiester oligonucleotide. By controlling the release of the oligonucleotide, the liposome formulation was able both to protect this molecule from the degradation and to prolong its residence time into the vitreous. The liposomes had also pronounced effect on the distribution of pdT16 in the posterior segment of the eye by reducing the amount of oligonucleotide in non targeted sites. Such a preferential pdT16 uptake by the retinal cells could opens interesting perspectives for using antisense phosphodiester oligonucleotides in the treatment of retinitis.

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CURRICULUM VITAE

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CURRICULUM VITAE

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March/ 2000 – PhD. In Pharmacotechnie et Biopharmacie de l'Université de Paris Sud (Paris XI) France

1994 –M.Sc. in Organis Chemistry – Instituto de Ciências Exatas (ICEx) da Universidade Federal de Minas Gerais (UFMG) – Belo Horizonte – Minas Gerais – Brazil

1989 – Pharmacist/ Specialist in Pharmaceutical Technology/Industry – School of Pharmacy–Universidade Federal de Minas Gerais –Brazil

3. Posts held

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1990-1991 – Pharmacist Chief of Pharmaceutical Production Service at Teuto-Brasileiro Pharmaceutical Laboratory-Contagem, Minas Gerais-Brazil.

4. Recent publications: List only the five most important publications over the last five years.

Mosqueira V, Legrand P, Gref R, Heurtault B, Appel M, Barratt G. (1999) Interactions between a macrophage cell line (J774 A1) and surface-modified poly(D,L-lactide) nanocapsules bearing poly(ethylene glycol). *J. Drug Targeting* 7 (1) 65-78.

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PHYSICO-CHEMICAL AND BIOLOGICAL EVALUATION OF THE NOVEL LONG-CIRCULATING BIODEGRADABLE NANOCAPSULES AND THEIR APPLICATION ON THE PARASITIC DISEASES THERAPY

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Introduction

The present work concerns the development of a new formulation of nanocapsules (NC) which can avoid phagocytosis and therefore remain longer in the circulation after intravenous administration. The physicochemical characterization of the NC [1] included an investigation of their properties, such as surface charge, size, hydrophilicity, morphology and homogeneity as a function of NC composition, nature of the hydrophobic polymer block, chain length and density of PEG. These properties of PEG-modified and unmodified NC were correlated with their interactions with biological components such as human serum, complement proteins and macrophages. The relationship between modifications of the surface of NC by adsorption or covalent grafting of polyethylene glycol (PEG) on complement activation (C3 cleavage) and on uptake by macrophages are discussed [2]. Complement activation was assessed by two-dimensional immunoelectrophoresis of complement component C3. Fluorescence microscopy of NC, labeled with a fluorescent oil dye, contributed to understanding the cellular pathways involved in NC interactions with phagocytic cells *in vitro* [3].

Furthermore, the *in vivo* distribution of these novel surface-modified biodegradable NC prepared from PLA-PEG copolymers with a range of PEG chain length and content were studied [4]. In order to validate the results, the stability of this new colloidal system in plasma was also studied. This is the first time that such physicochemical properties have been correlated with biological interactions for NC with a structure more complex than nanospheres.

Parasitic diseases therapy with Stealth® Nanocapsules

Conventional and surface-modified nanocapsules can be used to prepare intravenous formulations of antiparasitic drugs. Some perspectives of the use of NC formulations on protozoonoses therapy concerns their ability to circulate longer in the bloodstream and to increase the intracelular concentration of the drugs in target parasitized cells. The long-circulating properties of these NC and their ability to encapsulate a high payload of lipophilic drug in their oily core were exploited in order to prepare a original intravenous formulation of the antimalarial drug halofantrine (Hf). After physico-chemical characterisation and a study of drug release *in vitro*, the NC were tested against *Plasmodium berghei* in mice [5].

Experimental methods

Halofantrine chlorhydrate was purchased from SKB (UK). Different polymers were used: Poly(D,L-lactic acid) PLA50 (Mw) of 42000 g/mol was supplied by Phusis (France), poly(ε-caprolactone) (PεCL) 42500 g/mol and poly(D,L-lactide-co-glycolide) PLGA (75:25) wt ratio lactic/glycolic acid) 75000-120000g/mol by Sigma-Aldrich (France). PLA-PEG 45-5 (PLA Mw 45000 and PEG Mw 5000), PLA-PEG 45-20 (PLA Mw 45000 and PEG Mw 20000), PLA-PEG 2-5 (PLA Mw 2000 and PEG Mw 5000), PLGA-PEG 45-5 (PLGA 75:25 Mw 45000 and PEG Mw 5000), PεCL-PEG 45-5 (PεCL Mw 45000 and PEG Mw 5000) g/mol were synthesized and characterized as described [2]. Epikuron170 (70% soy phosphatidylcholine) was purchased from Lucas Meyer (France). Miglyol 810N was kindly provided by Hülls (Germany).

Nanocapsules preparation

The free base was prepared by treating the solution of Hf salts as described[5]. Nanocapsules were prepared with 6-10mg/ml of the polymer which were solubilized in acetone solution with 75mg of Epikuron170 and Miglyol 810N containing Hf as free base form (1mg/ml). This organic solution was poured into the external aqueous phase (with or without) Poloxamer 188 before evaporation of the acetone under vacuum. Drug content was analysed by HPLC.

Results

Monodisperse preparations of 200 nm in diameter were obtained by interfacial deposition of poly(D,L-lactide) with or without covalently bound polyethylene glycol (PEG). The effect of surface modification by PEG was assessed in several biological models. Both the activation of complement and capture by a macrophage-like cell line were reduced by covalently linked PEG, especially with longer PEG chains at a high grafting density.

The plasma clearance and biodistribution of different NC formulations were compared in mice, using radio-labelled PLA polymer. The plasma clearance was reduced 15 times for NC surface-modified by PEG of 20kD, compared with NC stabilised by adsorbed poloxamer 188. A considerable reduction in liver and spleen uptake was also observed for surface-modified NC.

The application of halofantrine NC on the antimalarial therapy was evaluated *in vivo* in mice highly infected with *P. berghei*. NC formulations of halofantrine showed to be at least as effective as the only other preparation described in the literature as well as being better tolerated. In the four-day test, PLA-PEG nanocapsules showed a slight advantage over conventional NC The more sustained antimalarial activity seen for the surface-modified NC could be explained by reduced massive mononuclear phagocyte system (MPS) uptake compared to unmodified nanocapsules. The pharmacokinetics of the drug were correlated with the pharmacological effect in heavily infected mice. The area under the plasma concentration-time curve was 6 times greater for NC-associated halofantrine than for a solubilised form of the drug. The parasitemia was reduced more rapidly with the Hf NC than with the Hf solution.

Conclusion

These results suggest that such NC could be used as an intravenous treatment for severe and cerebral malaria.

Acknowledgements

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APGI-01 *IN VITRO* INVESTIGATION OF THE INTERACTION BETWEEN LIPOSOMES AND METHYLATED β-CYCLODEXTRIN.

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Introduction

The final aim of this work is to design to a novel pharmaceutical dosage form composed of a dispersion of liposome-encapsulated peptide within a bioadhesive hydrogel containing methylβ-cyclodextrin to improve the absorption of the drug across the buccal cavity. Liposomes will be used to protect the peptide from enzymatic degradation and to create a sustained release effect; a bioadhesive polymer (Carbopol®974PNF) will be employed in order to prolong the time of contact between the liposomes (and thus also the peptide) and the buccal mucosa. Finally, methylβ-cyclodextrin, dissolved in the carbomer gel, will be used as an absorption enhancer. As methylβ-cyclodextrin is known to interact with the cell membrane components, especially cholesterol, this molecule could interact with liposomes inducing a destabilization of the vesicles. The main purpose of this work was to investigate the *in vitro* interaction between two formulations of liposomes, PC:CHOL:SA and PC:CHOL:DSPE-PEG_{2000} and methylated β-cyclodextrin.

Experimental methods

- **Materials**

BETA W7 M1.8, was a gift from Wacker-Chemie GmbH (Burghausen, Germany)
Soya bean Phosphatidylcholine (PC), Lipoid GmbH (Ludwigshafen, Germany)
Cholesterol, Grade: 99+%, Stearylamine and Inulin (Dahlia tubers), Sigma Chemical Co (St. Louis, MO, USA)
1,2-Distearoyl-sn-Glycero-3-Phosphoethanol-amine-N-[Poly-(ethyleneglycol)-2000] (DSPE-PEG), AVANTI®, Avanti Polar Lipids, inc. (Birmingham, AL, USA)
NET-314L INULIN, [^{3}H(G)]- (Dahlia tubers), specific activity 360.0 mCi/gram, NEN™ Life Science Products (Boston, USA)

- **Methods**

Liposome preparation

PC:CHOL:SA (molar ratio 60/30/10) and PC:CHOL:DSPE-PEG2000 (molar ratio 64/30/06) liposomes were produced by the method described by Bangham [1]. To calibrate the liposome diameter the suspensions were extruded, using an extrusion device, through polycarbonate membranes with diameters of 0.4µm and 0.2µm, respectively. A radiolabelled model compound, inulin (^{3}H) was encapsulated in the liposomes by the freeze/thaw technique. Free inulin was removed by ultracentrifugation of the vesicles.

Characterization of the liposomes

Measurement of diameter

The diameter of the liposomes was determined using a Nanosizer Coulter N4 Plus (Coulter Coultronics, Margency, France).

Measurement of zeta potential

The zeta potential was measured by a zetasizer 4 (Malvern Instruments, Orsay, France).

Lipid assay

The lipid dosage was performed with a kit (Phospholipides enzymatiques PAP 150). With the kit it is possible to measure the content of phosphatidylcholine, due to generation, by an enzymatic reaction, of a coloured product (quinoneimine), which can be determined quantitatively with a spectrometer (505 nm).

***In vitro* release study**

The *in vitro* release of inulin out of the liposomes (PC:CHOL: SA and PC: CHOL: DSPE-PEG) was carried out in 6ml vials placed in a thermo stated water bath at 37°C with lipid concentrations 2, 6 and 10 mM and methyl-β-cyclodextrin concentrations of 0%, 2% and 5%. After 30min., 6h and 24h of incubation, the samples were ultracentrifuged at 150 000g for 1.5h at 4°C and free inulin in the supernatant was measured by radioactivity.

Solubilization study

The experiments were carried out by a continuous addition of methyl-β-cyclodextrin solution to the liposome preparations and were analysed by turbidity. Turbidity was monitored at 37°C by measuring the absorbance at 400 nm, using a Perkin-Elmer Lambda 2 double beam spectrophotometer.

Results and discussion

The liposomes (OLV) for both formulations had a diameter close to 185 nm with zeta potentials of +55mV and –38mV for PC:CHOL:SA and

PC:CHOL:DSPE-PEG, respectively. A percentage of encapsulation around 10% and 7% and an efficacy of around 45µg inulin/µmol lipid and 20µg inulin/µmol lipid for PC:CHOL:SA and PC:CHOL:DSPE-PEG liposomes, were found respectively.

The *in vitro* release studies at 37°C with methyl-β-CD mixed with liposome suspension have shown, that for both liposome formulations, the release of inulin was dependent on the concentration of methyl-β-CD (Fig. 1 and 2). At high methyl-β-CD concentrations, an elevated release of inulin was observed. The concentration of lipid influenced the release of inulin. At increased lipid concentrations a less pronounced release took place. The liposome formulation had an impact on the amount and the release profile. The PC:CHOL:SA liposomes formulation (Fig. 1) demonstrated an instantaneous and almost complete release, which, consequently, did not increase considerably over time.

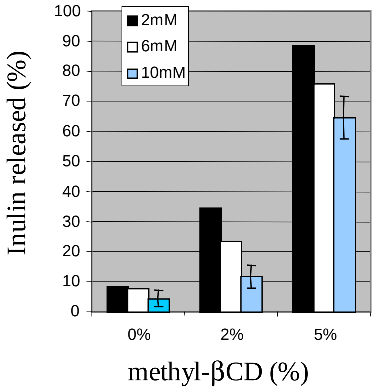

Fig. 1: Release of inulin (%) after 30 minutes at 37°C from PC:CHOL:SA as a function of the concentration (%) of methylβ-cyclodextrin at different lipid concentrations (mM) in the incubation media

The sterically stabilized PC:CHOL:DSPE-PEG liposomes (Fig. 2) show a different release pattern, with a less pronounced inulin release. The difference between the two formulations was most pronounced at the highest methyl-β-CD concentration (5%), where the amount of inulin released from the PC:CHOL:DSPE-PEG liposomes, even after 24 hours, was almost bisected compared to the release from the PC:CHOL:SA liposomes. Consequently, the sterically stabilized PC:CHOL:DSPE-PEG liposomes show a much higher resistance to interaction with methyl-β-CD, which is not persistent over time at low lipid concentrations.

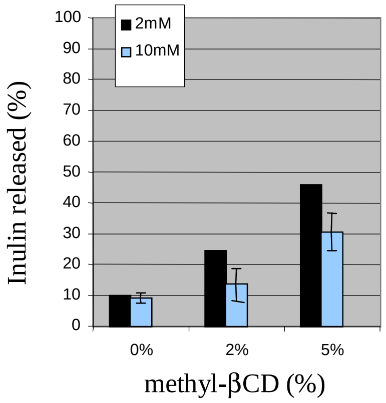

Fig. 2: Fig. 1: Release of inulin (%) after 30 minutes at 37°C from PC:CHOL:DSPE-PEG$_{2000}$ as a function of the concentration (%) of methylβ-cyclodextrin at different lipid concentrations (mM) in the incubation media.

The considerable release of inulin from the PC:CHOL:SA liposomes mixed with methyl-β-cyclodextrin (2% or 5%) after 30 minutes exclude a simple passive diffusion of inulin. A solubilization of the vesicles could occur.

An investigation of the exact mechanism responsible for the interaction between liposomes and methyl-β-CD was desirable. To evaluate the solubilising effect of methylβ–CD, experiments were carried out by a continuous addition of methylβ-cyclodextrin solution to liposome preparations and were analysed by turbidity measurements in the liposomes-methyl-β-CD-media. A dramatic decrease in absorbance is observed indicating that methyl-β-CD induced a solubilization of the vesicles depending on the lipid and cyclodextrin concentrations.

Conclusion

This work showed that methylβ–cyclodextrin interacted with liposomes inducing a solubilization of the vesicles. The dispersion of the liposomes within a bioadhesive hydrogel containing methyl-β-CD could modify the interaction intensity between the vesicles and the cyclodextrin due to the high viscosity of the gel. Next experiments will be performed in the presence of Carbopol®974PNF gel.

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APGI-02 *IN VITRO / IN VIVO* CHARACTERISATION OF THE FIRST PURE ANTIESTROGEN ASSOCIATED WITH LONG CIRCULATING BIODEGRADABLE DRUG CARRIER

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Introduction

The bioavaibility of orally given Tamoxifen (Tam), the leader antiestrogenic compound used in the treatment of breast cancer is very poor. In addition, Tam is a mixed agonist/antagonist which has a number of domageable side effects [1] and always lead to resistance after long term treatment. The pure antiestrogen RU [2] is able to induce up to 30% disappearance of MCF-7 breast cancer tumors implanted in nude mice and it inhibits Tam-induced resistance. In order to improve the bioavaibility of this pure antiestrogen we incorporated RU into long circulating biodegradable nanoparticles composed of different Polyethylenglycol (PEG) coated formulations made of different polyester cores (Polylactideacid or PLA, Polylactide-co-glycolideAcid or PLGA, Poly-ε-caprolactone or PCL).

Experimental Methods

. Preparation of PEG coated nanoparticles

The copolymers based on polyester-PEG were synthetised according to the method described by Gref et al. [3] and NS were prepared according to Fessi et al. [4].

. Nanoparticles characterization

The size of nanoparticles and zeta potential were measured by quasi-elastic laser light scattering with a Nanosizer N4 Plus (Coulter electronics, USA) and a Zetasizer (Malvern Instr., UK), respectively. The RU amount incorporated into the drug carriers was calculated by a HPLC method, as the difference between the total amount used to prepare the RU -loaded nanoparticles and the amount of free RU in the aqueous phase after centrifugation (30000g, 45 min).

. Opsonisation characterization and opsonin quantification

PEG layer-induced opsonin repulsion efficiency was measured after incubation for 30 min of nanoparticles into mouse serum following centrifugation (50 min, 30000g) and extensive washing with demineralized water. Nanoparticles bound proteins were desorbed by 1/5th vol containing 1% SDS. Proteins were quantified by a sensitive method using AmidoSchwartz dye[5] and further analyzed by SDS-PAGE (7.5 and 12.5% acrylamide) and silver staining Western Blot. These detections performed with antibodies against mouse IgG and C3b to allow a comparative measurement of the PEG coating efficiency as a function of the polyester used.

. In vivo evaluation of the antiuterotrophic activity of RU 58668 loaded PEG/PLA nanospheres

Immature female mouse (18 days old, 8 animals/group) were injected into the tail vein for 3 days with 100 µl of a suspension containing estradiol (E2) 5µg/kg/day + group1 : RU 100µg/kg/day in 5% glucose, or group2 : RU 58668 in PLA NP at 100µg/kg/day, or group3 : RU at 100µg/kg/day in PEG-PLA NP. After 3 days, (t=0, uteri weight =100%) each group received a single dose of E2 (5µg/kg) and the uteri were weighted at different times (6,16,36 and 48h)

Results

Table 1 : Characterization of different formulations of RU 58668 loaded-nanoparticles. (average values +/- SEM)

Nanoparticles	Mean Diameter (nm)	Zeta potential (mV)	Encapsulation Rate (%)
PLA [RU 58668]	173 ± 33	-70.6 ± 1.7	Up to 98%
PEG/PLA [RU 58668]	163 ± 30	-17.4 ± 1.9	99.3%
PCL [RU58668]	180 ± 45	-48.3 ± 1.5	Up to 98%
PEG/PCL [RU58668]	96 ± 38	-18.8 ± 2.8	98.97%
PLGA [RU 58668]	249 ± 64	-14.1 ± 0.4	94.10%
PEG/PLGA [RU 58668]	99 ± 38	-17.7 ± 2.7	99.8%

. Silver staining of SDS-PAGE and Western blotting showing the PEG-induced opsonin repulsion

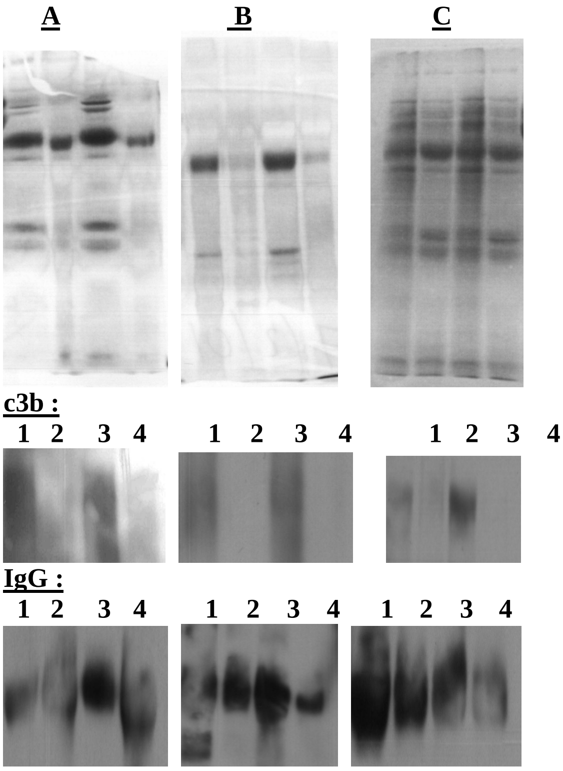

Figure 2 : Opsonin adsorption onto nanoparticles (A : NP with a PCL polymeric core, B : NP with a PLGA polymeric core, C : NP with a PLA polymeric core, 1 : NP unloaded without PEG coating, 2 : NP unloaded with PEG coating, 3 : NP loaded without PEG coating, 4 : NP loaded with PEG coating).
Upper panels A, B, C : Silver staining
Lower panels : Western blots

Silver staining of PAGE and Western blotting experiments revealed that PEG coupling onto NP's reduced significantly opsonin adsorption, since a decrease of protein amount was observed in the case of pegylated NP as compared to non peggylated ones.

. Prolonged antiuterotrophic activity of RU loaded NS PEG/PLA

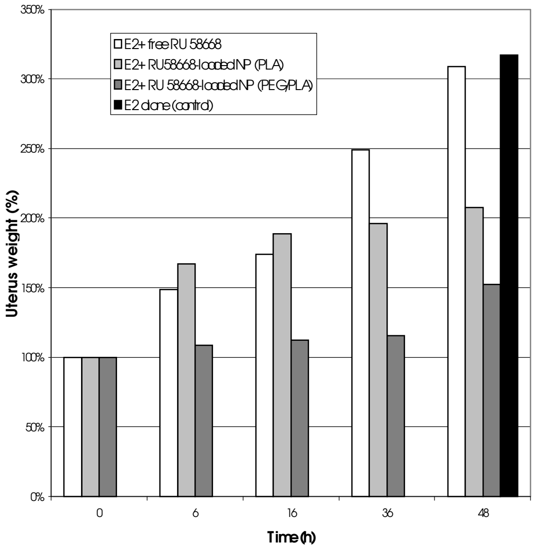

Figure 3 : Inhibition of uterotrophic activity in vivo induced by estradiol in female immature mice.

Conclusions

The small size PEG-coated polyesters NS (PLA in particular) have shown to incorporate the pure antiestrogen RU with a high yield leading to obtain nanospheres loaded with 38 μg PA/mg polymer. PEG coupling led to repulsion of opsonins as revealed by Western blot.Tested in immature mice RU incorporated into PEG coated PLA nanospheres revealed to prolong significantly its antiuterotrophic activity.

Acknowledgements

This work was supported by the ARC (n°9863 to JM Renoir) and the Ligue Nationale contre le Cancer (Comités du Cher, de l'Indre et des Yvelines).

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APGI-03 DNA-INDUCED LATERAL PHASE SEPARATION IN MIXED PHOSPHOLIPID LIPOSOMES DETECTED BY HIGH SENSITIVITY DIFFERENTIAL SCANNING CALORIMETRY

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Introduction

Recently, mixed phospholipid liposomes composed of a cationic lipid and a 'helper' lipid complexed with plasmid DNA have been used to deliver genetic material into eukaryotic cells [1]. The role of the cationic lipid is to provide electrostatic attraction between the liposome and the plasmid DNA and also with the negative cell wall. The neutral 'helper' lipid aids in delivering the DNA into the cell by promoting cell fusion [2]. However, its actual role is still not fully understood. A variety of techniques have been used to establish the morphology of the complexes produced upon mixing cationic liposomes with DNA, such as cryo-transmission electron microscopy (Cryo T.E.M) [3], synchrotron x-ray scattering [4] and atomic force microscopy [5]. The types of complexes formed depends on both the vesicle composition and the charge ratio of cationic lipid and DNA.

Although the structure and morphology of these complexes has been widely studied, little is known about the thermodynamics of such systems. Differential scanning calorimetry (DSC) has been used to study a wide variety of transitions in biological systems and has the advantage of requiring no immobilisation or modification of the reactants involved [6]. In this report we describe a study of the partial phase separation of the components of the liposome bilayer resulting from the electrostatic binding of the cationic lipid headgroups with plasmid DNA, using high sensitivity DSC.

Materials and Methods

A dry lipid film containing DOPE (L-α-dioleoyl-phosphatidylethanolamine (Sigma, UK) and EDMPC (ρ-ethyldimyristoylphosphatidylcholine (GlaxoSmith-Kline, UK) at various ratios (mol/mol) was hydrated using buffer (10mM Tris, pH 8) to produce multilamellar vesicles (5 mg/ml). Small unilamellar vesicles (SUVs) were produced by extrusion through polycarbonate filters of pore size 100 nm until a vesicle diameter of 100-130 nm had been produced. Plasmid DNA (9067 base pairs, GlaxoSmithKline, UK) was then added to the liposome dispersion to produce a final concentration of 0.55 :M and the resulting dispersion gently vortexed for 5 mins to aid mixing and left for 2 hours before use. HSDSC scans were taken using a MicroCal III (Seteram, France) equipped with twin-fill cells of 0.8 ml capacity for both the reference and sample cells at a scan rate of 1°C/min.

Results and Discussion

Upon cooling and heating pure EDMPC liposomes, an endothermic transition was seen at a temperature of 21.96 ± 0.3°C corresponding to the transition from the gel (L_β) to liquid crystalline (L_α) state. The low temperature shoulder is possibly due to the different species present within the liposomal bilayer membrane caused by the charge repulsion and different hydration levels between the individual lipids. The addition of the 'helper' lipid DOPE to the liposome formulation at different mol ratios has a pronounced effect on the endotherms produced (Figure 1).

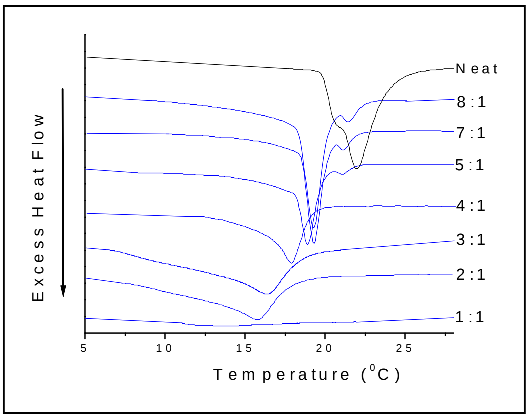

Figure 1. DSC thermograms of EDMPC liposomes containing various mol ratios of DOPE

Upon addition of DOPE at mol ratios 8:1, 7:1 and 5:1, the main phase transition decreases in temperature to 19.3 ± 0.3, 19.28 ± 0.3 and 18.81 ± 0.25°C respectively.

Due to its packing parameters= pure DOPE will not form bilayers and lipososmes under normal conditions, but will incorporate into bilayers if stabilised by a bilayer forming lipid such as DOPE. The inclusion of DOPE into the membrane effectively reduces the cooperativity of the main phase transition and lowers its temperature. The high temperature shoulder seen is possibly due to separate EDMPC phases being present due to the occurance of non-ideal mixing. This shoulder is abolished by incorporation of DOPE at higher mol ratios. As The ratio of DOPE to EDMPC is further increased, the main phase transition decreases in temperature and the endotherm broadens until no transition is seen at equimolar proportions. At this point the DOPE molecules are ideally mixed within the bilayer and result in the liposome remaining in a relatively fluid state at all temperatures.

The addition of plasmid DNA to the liposome suspension has a pronounced effect on the DSC thermograms produced. DSC thermograms of vesicles to which DNA has been added are shown in Fig 2.

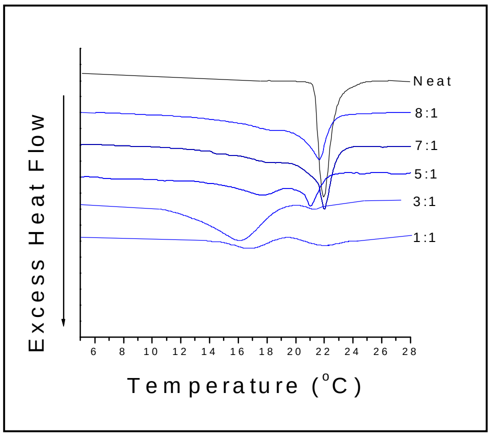

Figure 2. DSC thermograms of EDMPC/DOPE liposomes mixed with DNA

Upon addition of plasmid DNA to the liposomes, the main phase transition of all dispersions split into two distinct peaks, indicating that some degree of phase separation was taking place. The high temperature peak corresponds to an EDMPC enriched phase resulting from the molecular rearrangement of the phospholipids within the bilayer. The lower temperature peak corresponds to a more mixed EDMPC/DOPE phase brought about by the separation of the individual components.

Due to the separation of the phospholipids within the fluid plane of the bilayer, it is possible that some DOPE enriched domains are formed. However, it was not possible to measure these transitions as they are likely to occur outside of the temperature range investigated.

Conclusion

From these results it can be seen that the addition of plasmid DNA to a liposome suspension comprised of a cationic and ‘helper’ lipid greatly affected the thermal properties of the system. The electrostatic binding of the DNA results in a separation out of the components to form EDMPC enriched domains to which DNA is bound, unbound EDMPC/DOPE mixed phases and possibly DOPE enriched domains.

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APGI-04 RHEOLOGICAL COMPARISON BETWEEN CELLULOSE AND LOCUST BEAN GUM GELS

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Introduction

Sodium carboxymethylcellulose (CMC) and hydroxyethylcellulose (HEC) are two semi synthetic polymers traditionally used in pharmaceutical and cosmetic formulations. They are used as binders in tablets and as thickening agents in many others products. Locust bean gum (LBG) or *Ceratonia siliqua L.* gum is a natural polymer extracted from carob tree seeds, spontaneous in Mediterranean area. It is also used in pharmaceutical formulations, although in a smaller scale. These three polymers are nonionic and water soluble. Rheological characterization of such polymeric materials can be of importance as provides fundamental information for the assessment of some of the final properties of a formulation, where any one is included, such as storage stability or consistency. The release of an entrapped medicament is also affected by the viscosity of a formulation [1]. The aim of this work is to compare these polymers regarding their rheological properties. For this purpose were prepared gels of each polymer in three different concentrations and their rheological behaviour was established by continuous shear rheometry.

Experimental methods

- **Materials**

Sodium carboxymethylcellulose (HV) and hydroxyethylcellulose were obtained from BDH CHEMICALS and HENKEL respectively. Locust bean gum HG M175 was prepared by INDAL, Industrias de Alfarroba Ld. Faro.

The preservatives, nipagine and nipasol were obtained from MERCK.

Distilled and dionized water was obtained using a Mili-Q system.

- **Gels preparation**

Three gels of different concentrations of each polymer were prepared.

1.25%, 1% and 0.75% (w/w) gels of sodium carboxymethylcellulose and locust bean gum were obtained by mixing the appropriate calculated amounts of the polymers in boiling water contained nipagine and nipasol. Stirring using the ULTRA TURRAX mixer was maintained untill a cold clear gel was formed.

For hidroxyethylcellulose gels at 1.5%, 1.25% and 1% (w/w) a similar proceeding was performed.

- **Rheological measurements**

The rheograms and the flow curves of the different hydrogels were achieved using a rotational rheometer with 30 speeds – RHEOMAT 30 supplied by CONTRAVES A.G. Zurich. Its co-axial cylindrical measuring system is named MS-DIN 53788 (45/8). The gels are introduced between an external settled cylinder and an internal movable cylinder. Measurements of torque reaction due to shear stress (τ) for the 30 different rates of shear (ε), were made using Model nº25 at 22ºC temperature. The viscosity values (η) for corresponding shear stress were obtained by a conversion table.

Results and discussion

With the purpose to establish rheological comparison between the three polymers a previous study was made to choose the concentration values used in the present work [2].

The gels at different concentrations are shear thinnings, with pseudoplastic behaviour.

They do not exhibit significant yield value or histeresis on their rheograms and the apparent viscosity increases along with the concentration of the gels.

For the materials exhibiting pseudoplastic behaviour no single value of viscosity can be considered as characteristic. However at higher shear stresses, the flow curves tend toward linearity indicating that a minimum viscosity can been attained. Such a viscosity can be an useful mean of classification and comparison.

So, the relationship which is most frequently used for pseudoplastic products is given by the equation [3]:

$$\tau = \eta\ \varepsilon^n$$

τ - shear stress, ε - shear rate, η - viscosity coefficient and the exponent n is the index of pseudoplasticity.

$$\text{So, } \log \tau = \log \eta + n \log \varepsilon.$$

This equation, equivalent to an equation of a straight line, can be only used over limited ranges of shear stresses.

In all viscosity curves obtained (Fig. 1, 2 and 3) there are 12 points (n') on linear range.

In order to obtain the values of the constants, used for comparison, the slope n and the intercept log η, log ε was plotted against log τ (Fig. 4, 5 e 6).

The equation to obtain the slopes of straight lines is:

$$n = \frac{\sum xi \sum yi - n' \sum xi\, yi}{(\varepsilon \sum xi)2 - n' \sum xi\ 2}$$

(for each point i, log ε is xi and log τ is yi).

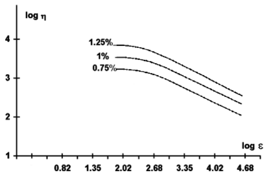

fig.1

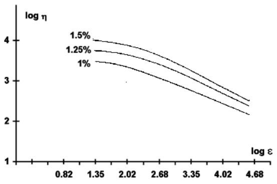

fig.2

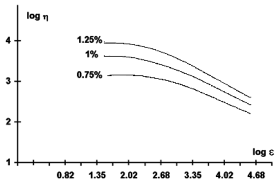

fig.3

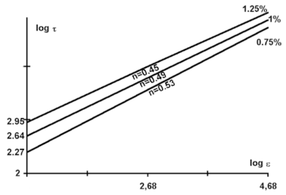

fig.4

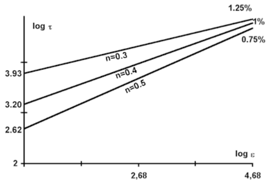

fig.5

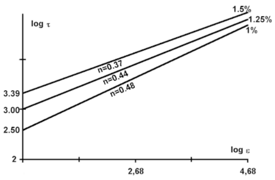

fig.6

Figs. 1,2,and 3 – Viscosity curves of LBG, HEC and CMC gels, respectively.

Figs. 4,5 and 6 – Rheograms of CMC, HEC and LBG gels, respectively.

Table 1
Values of the different constants determined for each gel concentration

Polymers	Gel concent. (%)	n	Intercept Log η
Locust bean gum	0.75	0.50	2.62
	1.00	0.40	3.20
	1.25	0.30	3.93
Sodium Carboxymethylcellulose	0.75	0.53	2.27
	1.00	0.49	2.64
	1.25	0.45	2.95
Hidroxyethylcellulose	1.00	0.48	2.50
	1.25	0.44	3.00
	1.50	0.37	3.39

Conclusion

It is possible to obtain formulations containing any one of the three polymers with similar rheological properties if appropriate concentrations are chosen. For example, the rheological behaviour of a 0.75 % locust bean gum gel is similar to the rheological behaviour of 1% gels of both cellulose polymers (table 1).

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APGI-05 THE USE OF MTDSC AND GPC TO STUDY THE DEGRADATION BEHAVIOUR OF PLA MICROSPHERES

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Introduction

Poly (d, l – lactide) PLA is a copolymer of two stereoisomers, D- and L-lactic acid. Due to its amorphous nature it degrades much faster than the homopolymers of D- or L- Lactic acid, which have crystalline structures [1]. PLA has been used for the controlled release of pharmaceutical drugs because of its biocompapatible and biodegradable properties. Drugs encapsulated within these microspheres have been used as injectable controlled delivery release systems, which do not require removal after the drug has been depleted [2]. PLA degrades into natural by-products of the body and is excreted as lactic acid. It is believed that these microspheres degrade homogeneously by bulk erosion, although studies have also shown heterogeneous degradation of large particles [3]. The objective of this study was to determine whether these PLA microspheres undergo homogeneous or heterogeneous degradation and investigate the effect of 0.1*M* phosphate buffer saline (PBS) on the glass transition temperature of PLA microspheres which are dehydrated (dried in oven at 37 C for 5 days) and hydrated (un-dried analysed straight from PBS).

Experimental methods

- **Materials**

The following materials were received from commercial suppliers: Sigma, Poole, UK supplied PLA Mw 106,000. BDH, Poole supplied polyvinyl alcohol Mw 22,000, potassium dihydrogen orthophosphate and sodium hydroxide solution. PLA Microspheres with a mean volume median diameter of 7.7 μm were prepared using a solvent evaporation technique. 500 mg of PLA was dissolved in 10 mL of dichloromethane. The solution was poured into 125 mL of 2% w/v polyvinyl alcohol solution and homogenised for 2 minutes at 8000 rpm. The resulting emulsion was stirred continuously for 20 hours with an overhead stirrer at 500 rpm. Once microspheres had formed, they were incubated in 0.1*M* PBS pH 7.4 and agitated at 40 rpm in a water-bath at 37°C. At predetermined intervals the microspheres were isolated from PBS and analysed. The surface morphology was observed by scanning electron microscopy (SEM) (Philips XL20) Particle size was measured by laser diffraction (Malvern Mastersizer SX). Mass loss was determined gravimetrically; weight loss was determined by gel permeation chromatography (GPC). Modulated temperature differential scanning calorimetry (MTDSC) studies were also performed to analyse PLA's glass transition temperature when hydrated (TgH) and dehydrated (TgD) (TA Instruments 2920).

Results and discussion

Table 1.

Effect of degradation on mass, molecular weight and glass transition of PLA incubated at 37ºC and pH7.4 (mean ± SD = 3).

Day	Mass Loss (%)	Molecular Weight (Mw)	Poly dispersity (Pd)	Tg H (°C)	Tg D (°C)
0	0	112100 ±2185	1.9	55.33 ±0.42	55.33 ±0.42
10	13.69 ±3.3	95400 ±2600	1.9	51.42 ±0.56	53.59 ±0.25
20	11.74 ±4.0	86600 ±1990	2.2	40.88 ±0.04	50.81 ±0.42
30	15.81 ±2.7	82990 ±2365	1.7	40.99 ±0.26	51.63 ±1.41
40	17.17 ±3.0	75700 ±1900	1.8	40.81 ±0.33	51.91 ±1.82
50	19.57 ±1.4	75700 ±3300	1.8	-	-
60	23.60 ±8.8	75400 ±2100	1.6	40.61 ±0.38	51.46 ±0.19
70	30.65 ±1.7	68700 ±2100	1.6	40.48 ±0.67	51.23 ±0.28
80	29.93 ±4.4	67700 ±1500	1.6	42.50 ±0.37	51.72 ±0.79
90	30.13 ±3.3	65000 ±2200	1.5	42.71 ±0.34	51.56 ±0.50
100	29.29 ±5.2	61000 ±490	1.5	41.30 ±1.01	51.72 ±0.37

As shown in table 1 as that the length of incubation time increases the microspheres mass loss and weight loss increased, whilst the glass transition of the hydrated and dehydrated micropsheres decreased to an extent. The initial glass transition (T_g) of PLA was approximately 55°C. By day 100 the hydrated microspheres T_g had dropped to approximately 42°C. The decrease in T_g became virtually constant as from day 20, which could imply there is a limit to the plastisciation effect of PBS on the PLA microspheres. The dehydrated microspheres on the other hand showed a small decrease in T_g though the effect was less forthe hydrated PLA microspheres. The T_g of the dehydrated PLA microspheres dropped to approximately 52°C by day 100. The T_g of the dehydrated PLA microspheres became constant from day 20. The T_g temperature did not revert back to the initial T_g temperature of 55°C after drying. This may be an indication of the onset of degradation. Both hydrated and dehydrated PLA microspheres were in the glassy state since the T_g temperature did not drop below 37°C. In the glassy sate the mobility of the chains are restricted, so high molecular weight PLA microspheres would have slow diffusion of PBS.

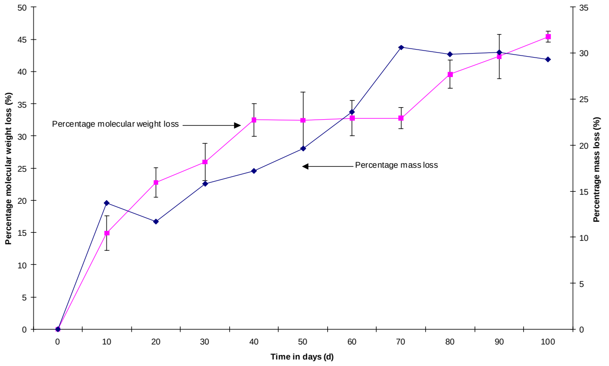

Figure 1. Percentage mass and molecular weight loss changes, of PLA microspheres in 0.1M PBS pH7.4 at 37^{o}C (mean ± SD = 3).

In Figure 1 there is an apparent relationship between the percentage molecular weight loss and percentage mass loss. As percentage molecular weight loss increased so did the percentage mass loss. By the end of the 100 days the percentage mass had dropped by 30% and the molecular weight had dropped by 45%. This indicates molecular weight loss occurs more rapidly than mass loss in PLA microspheres. Figure 2 shows the molecular weight change with time. The PLA microspheres show an apparent triphasic degradation profile. An initial accelerated phase of 40 days with a hydrolysis rate of 4.7× 10^{-3} d^{-1}, followed by a relatively stationary phase with a hydrolysis rate of 8.9 × 10^{-5} d^{-1} and a final but slower accelerated phase with a hydrolysis rate of 2.3 × 10^{-3} d^{-1}at day 60. This profile could be interpreted as rapid surface erosion, followed by slow penetration of buffer into the microspheres followed by fast bond cleavage. SEM studies revealed microspheres retained their original shape and had a slightly rough surface with no pores.

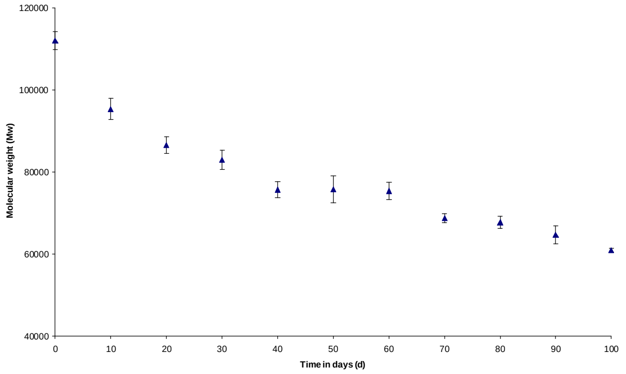

Figure 2. The change in molecular weight of PLA microspheres with time (mean ± SD = 3).

Conclusion

GPC studies indicate that there may be more than one phase of degradation of PLA microspheres. Work done by Reich [3] suggests several phases exist and there is some evidence of this in the present study The MDSC studies show there is a limit to PLA plasticisation in PBS over100 days. SEM and GPC studies show PLA microspheres maintain their original shape, but steadily lose mass during degradation, which implies these microspheres are undergoing homogeneous degradation.

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APGI-06 CONJUGATES OF SOYBEAN PROTEASE INHIBITOR (BOWMAN-BIRK TYPE) WITH FATTY ACIDS: UPTAKE BY CACO-2 CELLS AND TRANSEPITHELIAL TRANSPORT

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Introduction

The soybean Bowman-Birk protease inhibitor (BBI) is currently receiving particular attention because of its anticarcinogenic activity. The *in vivo* transport of BBI to target tissues is limited by epithelial and endothelial barriers.

It has been recognized that absorption and penetrating through the cell membrane is often the rate-limiting step for systematic delivery of proteins and peptides. One of the attractive approach for improving the transport properties of proteins is the conjugation of them with membrane-binding carrier ligands. Fatty acids are the most useful type of such carriers and evidence suggests that fatty acid-conjugated proteins may be able to cross cell membranes.

The aim of the present work is to study the effect of BBI conjugation with various fatty acids on the uptake of these derivatives by Caco-2 cell monolayers and transepithelial transport.

Experimental methods

- **Conjugation of BBI with various fatty acids**

Fatty acid derivatives of BBI were synthesized using previously optimized procedures [1] with protection of the inhibitor active site.

Briefly, acylation with N-hydroxysuccinimide esters of oleic (Ole-), linoleic (Lin-) and α-linolenic (α-Lin) acids was carried out in the organic solvents (dimethylsulfoxide - dimethylformamide - pyridine). The acylated derivatives of BBI were purified from the native protein either by chromatography on DEAE-Sephadex or by precipitation at various pH. The yield of preparations was 65-75% .

- **The uptake of inhibitors by Caco-2 cells**

The native BBI and its conjugates were iodinated with ^{125}I by the chloramine-T method. Caco-2 cells were grown on 6-well cluster plates in DMEM medium supplemented with 10% fetal bovine serum. Confluent cell monolayers were treated with ^{125}I-BBI or ^{125}I-BBI-fatty acid derivatives in serum-free medium for 1 hr, and washed extensively using ice-cold PBS. Cell monolayers were trysinized, detached, and centrifuged, and cell pellets were washed three times with cold PBS and counted in a gamma counter. Subsequently, cell pellets were dissolved in 1N NaOH and the protein contents were determined by using a BCA protein assay kit (Pierce). Results from the cellular uptake were presented as ng BBI per mg cell protein.

- **Transport studies**

The transepithelial transport of native and acylated BBI was measured using filter-grown Caco-2 cells in 6-well cluster plates of Transwells. Cell monolayers were used at the 7th day after reaching a transepithelial electric resistance (TEER) of 750 ohm.cm^2. The radioactively labeled BBI or its derivatives was added to the apical medium, and aliquots of basal medium were removed at various time points. The radioactivity in the basal medium was determined using gamma counter. Results were presented as ng of BBI transported per monolayer at different time point.

Results and discussion

The modification of one amino group did not influence the inhibiting activity if BBI with previous reversible protected active site was used. The increase in the number of acylated NH$_2$-groups decreased the protein activity, (Table 1).

The cellular uptake of (Ole)$_1$-BBI, (Ole)$_2$-BBI, (Lin)$_2$-BBI and (α-Lin)$_2$-BBI by Caco-2 cells was

200-, 100-, 30-, 4-fold compared to the native inhibitor, respectively (Figure 1). It was demonstrated that the $(Ole)_1$-BBI had the highest affinity to Caco-2 cells. May be the difference in uptake between mono- and di-derivatives of BBI due to the organization some large aggregates by BBI-conjugates with two acylated NH_2-groups, which could prevent the interaction with cell monolayers.

The transepithelial transport of BBI-derivatives with modified NH_2-groups across Caco-2 cells was 6-9-fold in comparison with native BBI (Figure 2).

Conclusions

The procedure for preparation of homogeneous BBI-fatty acid conjugates with definit number of acylated NH_2-groups was developed.
This modification markedly increased the uptake and transport of the acylated BBI-conjugates across the Caco-2 cells. Among all conjugates in this study, $(Ole)_1$-BBI demonstrated the highest uptake by Caco-2 cells and transepithelial transport. Therefore, the acylation of proteins by fatty acids may provide a high affinity and a rapid mobility of the modified proteins in cell membranes.

Table 1
The main characteristic of BBI-containing derivatives

Sample	AntyTr. activity, %	AntyChym. activity, %	Separation of the native and acylated BBI
BBI	100	100	—
$(Ole)_1$BBI	78	85	Chromato-graphy
$(Ole)_2$BBI	71	80	Precipitation (pH 3.7)
$(Lin)_1$BBI	80	85	Chromato-graphy
$(Lin)_2$BBI	70	80	Precipitation (pH 2.9)
$(\alpha$-$Lin)_1$BBI	76	85	Chromato-graphy
$(\alpha$-$Lin)_2$BBI	70	79	Chromato-graphy

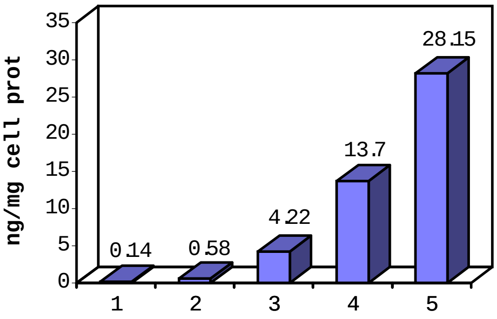

Figure 1. Uptake of proteins by Caco-2 cells (ng/mg cell protein). 1-BBI, 2-$(\alpha$-$lin)_2$-BBI, 3-$(lin)_2$-BBI, 4-$(Ole)_2$-BBI, 5- $(Ole)_1$-BBI.

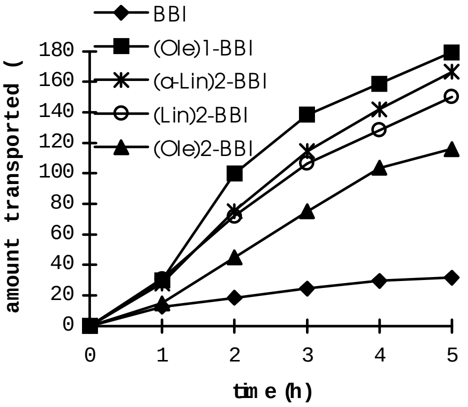

Figure 2. Transepithelial transport of BBI and its conjugates in Caco-2 monolayers

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APGI-07 FORMULATION OF INTERLEUKINE-2 STEALTH LIPOSOMES TARGETING CELLS OF THE IMMUNE SYSTEM

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Introduction

Interleukine-2 has been extensively investigated because of its central role in the regulation of the immune response by stimulating lymphocyte proliferation, T cell activation and antibody production by B cells. The production of recombinant (rIL-2) has cleared the way for large-scale applications, notably in oncology and AIDS treatment. However, rIL-2 is a 15-kDa protein which is rapidly eliminated by renal filtration and serious side effects, including capillary leak syndrome, are associated with systemic administration of the high doses of rIL-2 necessary to achieve effective concentrations.

To increase efficiency and reduce side effects, liposomes have been employed to increase the biological half-life of rIL-2. To avoid rapid elimination of liposomes, polyethylene glycol (PEG) can be grafted onto the surface. We studied a new vector to target immunocompetent cells. rIL-2 was attached to distal ends of PEG chains by a carbamate bound in order to provide targeting to lymphocytes.

These liposomes could be recognised by cells which express the IL-2 receptor. IL-2 included within the liposomes would be released near these cells which would be activated. We could obtain a new system more efficient and specific than "conventional" liposomes.

Experimental methods

• Materials

Dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylethanolamine (DSPE), distearoylphosphatidylethanolamine polyethyleneglycol (DSPE-PEG) were purchased from Avanti Polar Lipids, Alabaster, USA. Egg phosphatidylethanolamine were obtained from Lipoïd KG, Ludwigshafen, Germany. Interleukine-2 were supplied by Roussel-Uclaf (France). Calcein and bis(4-nitrophenyl carbonate) polyethylene glycol (2pNP-PEG) were purchased from Sigma, St Louis, USA. Human IL-2 Kit Duoset® and Substrate Reagent Pack were obtained from R&D, Minneapolis, USA.

• Permeability of calcein-loaded liposomes after 5 days

Lipids (10 mg DPPC or 10.5 mg EPC) with or without cholesterol (2.6 mg) were dissolved in chloroform. A film was obtained after drying in a dessicator and film was hydrated with a solution of calcein 40 mM with or without IL-2. This suspension was homogenized in an ultrasound bath for 1 min. To obtain calibrated multilamellar vesicles, an extruder with a 0.2-µm polycarbonate filter was used. The release of calcein in Tris buffer was observed by fluorescence spectrophotometry (excitation : 488nm; emission : 511nm) after three purifications on a PD 10 column.

• Proliferation activity of IL-2 subjected to different formulation conditions

IL-2 was subjected to different treatments corresponding to formulation conditions (temperature, bath and probe sonication). Then, it was incubated with an IL-2 dependent T lymphocyte cell line (CTLL-2). After 3 days of incubation, a MTT test was carried out to evaluate the number of viable cells.

• Liposome preparation

The film was composed of EPC : DSPE-PEG (18 : 1) in a chloroform solution and dried overnight. An IL-2 solution (250µg.mL^{-1}) was used to hydrate the lipid film. The suspension was mixed with glass beads in a vortex. The sample was then extruded with 0.8-0.4-0.2-0.1-0.05 µm polycarbonate filters. Calibrated liposomes were then separated by exclusion chromatography (Sephadex® G75). Liposomes were detected by light scattering and IL-2 by fluorescence emission (320nm) after excitation at 290nm.

• IL-2 release from PEG-bearing liposomes

IL-2 liposomes were prepared and separated as described above. Liposomes were incubated in pH 7.4 buffer. A sample of this suspension was then centrifuged on Microcon® 100 YM units (Millipore, France) to separate liposomes from released IL-2. IL-2 recovered in the filtrate was determined by a human IL-2 ELISA kit.

• Synthesis of pNP-PEG-DSPE

pNP-PEG-DSPE was synthesized as described by Torchilin et al. [1], by mixing DSPE in chloroform with triethylamine and a large excess of 2pNP-PEG (Mw= 3 400). Solvents were removed by evaporation. Micelles of pNP-PEG-DSPE were formed by hydratation and were separated from unbound PEG and 2pNP-PEG by exclusion chromatography. pNP-PEG-DSPE were then extracted with chloroform.

• Coupling of proteins to liposomes

Liposomes composed of EPC, DSPE-PEG, pNP-DSPE-PEG (18 : 0.6 : 0.4) were formulated as described previously, at acid pH to avoid hydrolysis of the para-nitrophenyl ester. IL-2 was added to the liposome suspension and pH was raised to 8.5. After incubation, liposomes were purified by exclusion chromatography.

• *In vitro* activity

Liposomes with only IL-2 on the surface were tested on CTLL-2 cells to evaluate the activity of the IL-2 and its capacity to recognize its receptor.

Results

To avoid a rapid release of IL-2, liposomes with minimal permeability must be chosen. Liposomes composed of DPPC and cholesterol showed the lowest calcein release (Fig. 1). However, the presence of cholesterol reduces the amount of IL-2 associated with liposomes (data not shown). As a best compromise, EPC liposomes were chosen.

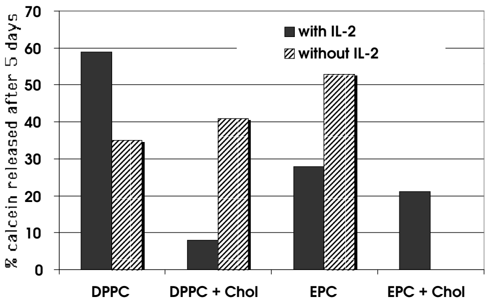

Figure 1 : Liposome permeability to calcein.

The effects of formulation conditions on IL-2 integrity was tested. Fig. 2A shows that IL-2 was sensitive to high temperature and to ultrasound produced by an ultrasound bath. These results indicate that lipids with a low transition temperature, such as EPC, should be used. Thus both permeability and biological studies justify the choice of EPC. Furthermore, the results in Fig. 2 show that extrusion rather than sonication should be used to reduce liposome size.

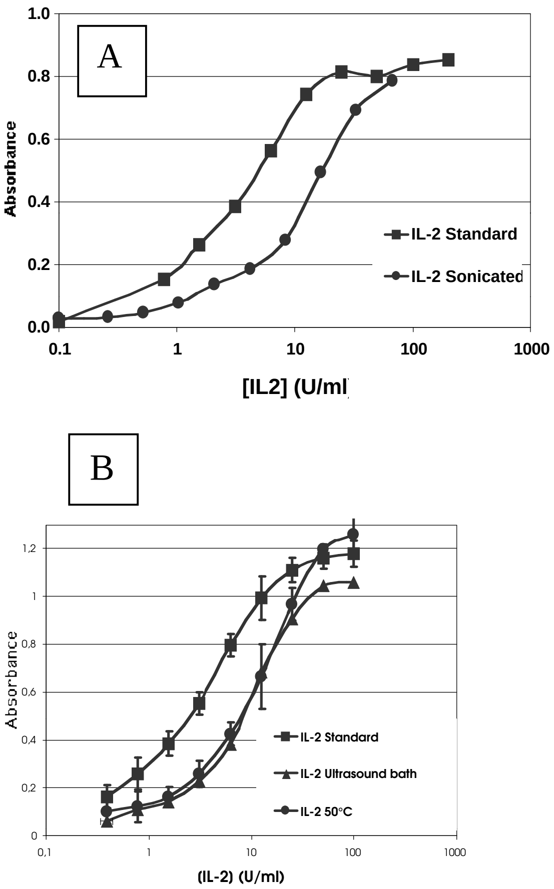

Figure 2 (A & B) : CTLL-2 proliferation curves representing IL-2 activity after different treatments

Conclusion

We could formulate liposomes with a longer half-life than "conventional" one and target immunocompetent cells. IL-2 released in the vincinity of these cells would be more active than free IL-2, thus allowing lower doses and reducing side effects.

Acknowledgement

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III Meeting of Pharmaceutical Quality Control

ENECQ

THE ALTERNATIVE TOXICOLOGY. THE CELL AS ONE OF THE MOST IMPORTANT PROTAGONIST.

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The Toxicology is the Science that studies among other aspects the potentiality of producing toxic effects in man, experimental animals and environment by chemical substances. The same as most of the branches of the knowledge it has had a tremendous development starting from the second half of last century, and begins with the principle of 3ERRES that constitutes the rationale of what today is know as Alternative Toxicology. The Alternative Toxicology is based in the procedures that Reduce, Refine and Replace animals in the Biomedical experimentations, the teaching and the regulatory and toxicological studies. The cell, as structural and functional basic unit of the human body is also playing a very important role in the evolution of this Science. Its employment is in numerous procedures developed by Institutions so noted as the ECVAM (European Center for the Validation of Alternative Methods), the CAAT (Center for Alternative Animal Testing), the ERGATT (European Research Group for Alternatives in Toxicity Testing), Some of these institutions have protocols worked out with those but refined validation procedures, based on the GLP (Good Laboratory Practice), to mention some of the most important ones. The assays that have been described and use the cell or the cellular cultures have been proposed to substitute test very well known like the Draize test, that determines the potential irritating effect of chemical substances on the ocular structures. Among these are also for example the one that uses the Red Blood Cells (RBC), or the studies of Photoirritation carried out on the shaved skin of animals exposed to the UV radiation, using erytrocytes. Assay of reception of the neutral red coloring for the 3T3 cells of mouse fibroblasts to demonstrate the cytotoxicity of some products after the exposure of the UVA radiation, this procedure has been approved by the European Union for their use inside the countries members and it is under study by the OECD (Organization Economical for the Cooperation and Development) for their acceptance. Another alternative method is the use of the cytotoxic effects that produce some substances over the cells L929, in order to study the Acute Toxicity (Undue) described in International Pharmacopeia for the evaluation of the security of the ¨Medical Devices¨ and approved by the Directive 93/42/EEC. Also is known the use of the cells Hep 2 to study the cytotoxic effect of implantable materials used in Medicine and Stomatology to only mention some examples. In our conference we will address the procedures currently used in view of substituting the traditional assays used in toxicology, the actual state of research in this field, and we will also show some results obtained in our laboratory using the procedures of RBC, the Photohemolisis, and that of the L 929 cells to detect possible harmful effects of chemical substances, that demonstrate convincingly that the cell is one of the main protagonist in the development of the Alternative Toxicology.

REFERENCE STANDARDS

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CELL CULTURE IN TOXICOLOGICAL ASSAYS

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For the past 10-15 years, there have been increasing acceptance and use of cell culture systems to investigate mechanisms of chemical-induced toxicity. Over the same time period, many cellular toxicity assays have also been developed for providing quantitative information on the general toxicities of chemicals. The reasons for developing in vitro cytotoxicity assays are the replacement of unethical and uneconomical animal tests, and also the extension of toxicity testing in various ways. Numerous advantages of in vitro systems such as speed, reproducibility of test conditions, increased sensitivity and economy in funds and animals are apparent. Despite these advantages a number of technical problems must still be addressed before there systems can be more fully utilized as replacements.

It is unlikely that cytotoxicological *in vitro* systems will ever provide toxicity data that will be totally equivalent to those derived from *in vivo* systems – just as the results of animal testing are note fully representative of the human experience of toxic exposure. It will be necessary for scientists to demonstrate that new techniques designed to replace existing assay system provide data that are in close agreement with data obtained with the established assay systems. In cases where the older test method produces numerous erroneous or poorly quantified results, the alternative assay need only demonstrate a significant reduction in the false-positive data yielded by the original test, in addition to a significant level of concurrence.

There are two examples of old assays as the LD50 for predicting acute lethal toxicity and the Draize rabbit test, which continue to be the methods of choice for the regulatory assessment of acute lethal toxicity and eye or skin irritation, respectively, of a wide range of chemicals including pharmaceuticals, cosmetics and their raw materials. However, the two tests have been exposed to public criticism because of animal welfare concerns..

Various types of alternative methods are being used for assessment acute lethal toxicity and potential eye or dermal irritation, including *in vitro* cell lines, primary monolayer cultures, skin slices, organ culture, reconstituted human skin equivalents and structure-activity relationship models. The *in vitro* tests incorporate numerous endpoints, including cell morphology, viability, membrane damage, metabolic effect and the release of various inflammatory mediators.

Any *in vitro* system used to evaluate a specific cytotoxic effect must be capable of displaying the toxic process caused by the test agent. A commonly used endpoint is cell death, which is an *all-or-none* phenomena. Agents that affect specific metabolic or enzymatic systems without causing cell death may still be quite toxic to an organism thus producing false-negative toxicological data if cell death is the only criterion of the endpoint. The frequently proposed solution in cell culture is to develop a battery of *in vitro* endpoints which, when considered as a unit, will reflect a variety of specific toxic mechanisms,

However, the amount of new alternatives, no test, combination of tests, or testing strategies has yet been developed which meets all the requirements of the regulatory authorities.

In this review we will present the different methodologies employed as alternative to the toxicological assays, based on cell culture, the situation of different validation studies of these methodologies and the strategies developed to study the toxicity of chemicals without animals

IN VITRO DISSOLUTION STUDIES

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Introduction

Dissolution is a critical parameter of pharmaceutical dosage forms. It is now well recognized that in vitro dissolution testing, whether in development or in circulation, is of importance to screen formulations during development and to ensure batch-to-batch quality control. In the USA and in Europe, more than 30 years of research have been devoted to characterizing the biopharmaceutical properties of drug products. Several guidelines have been published and all pharmacopoeias include a description of dissolution testing.

During drug development *in vitro* dissolution testing is an important tool in the evaluation of the best formulation and also in the understanding of possible risks related to specific gastrointestinal environment, dose dumping, food effects on bioavailability or interaction with other drugs. Today, the dissolution studies are the most frequently used tools in the development, characterization and utilization process of controlled release formulations.

Dissolution testing devices

Dissolution rate may be defined as the amount of active ingredient in a solid dosage form dissolved in unit time under standardized conditions of liquid/solid interface, temperature, and media composition. The *in vitro* release study of the drug from a dosage form can be performed using official methods described in the pharmacopoeias (European Pharmacopoeia, USP 24). Numerous factors affect dissolution, thus the dissolution medium, agitation, temperature are carefully controlled. The apparatus and specifications may be easily found however there is a wide variety of methods based on other apparatus. These are used because they may be faster, cheaper, easier, more sensitive to a particular problem for a particular drug, or developed by a particular investigator. The status of the solid/liquid interface is the key parameter for dissolution : it is the point at which fresh solvent comes into contact with the surface of the solid to be dissolved. An in vitro system should ideally maintain a sink condition and the dissolving solid should be bathed in fresh solvent.

The hydrodynamic conditions in an in vitro dissolution experiment can affect the dissolution in several different ways. The hydrodynamic conditions, defined by the design of the device, agitation intensity, flow, volume and viscosity of the medium, have to be well studied and standardized. It is generally accepted nowadays that alternative procedures to the beaker methods, such as the flow through method for example are necessary for determining the biopharmaceutical characteristics of a given product. According to the design of the drug dosage form (immediate or extended release, route of administration) and the drug studied, a specific dissolution test has to be devised and validated methods have to be developed.

Development of an *in vitro* dissolution method

Information about the in vitro dissolution characteristics of drug substances is important in order to establish the most suitable dosage form design for a given pharmaceutical product. If reliable in vitro dissolution methods can be identified, they may also serve as tools for studying batch to batch variability in order to establish adequate raw material specifications in term of in vitro dissolution properties.

Dissolution test development is heavily influenced by pharmacopoeial and regulatory guidances. A dissolution procedure consists of

- ☐ Selection of a dissolution apparatus
- ☐ Selection of dissolution media and decision on deaeration
- ☐ Selection of time points to collect samples and decision of what specification to set
- ☐ Development of a procedure to measure drug in dissolution fluid

For immediate release dosage forms, the Biopharmaceutics Classification Scheme can be used as a starting point to select suitable dissolution test conditions for a new drug. For class 1 and 3 compounds, the medium and method should be simple. For class 2 compounds, the choice of test will depend on the objective to be met.

For extended release dosage forms, solubility characteristics and the physiological environment have to be considered. Because of the numerous concepts of release developed by the industry, standard conditions for extended release formulations can be set.

Dissolution apparatus and methodology used for studying immediate and extended release formulations have to be validated. Dissolution testing devices must be calibrated, deaeration procedure and analytical drug assay must be validated.

Analysis of drug dissolution data

Apart from providing specifications to ensure batch-to-batch consistency within a range that guarantee acceptable in vivo performance and to determine limits between good and bad batches, drug dissolution data can give many more information.

Characterization of the complete dissolution profile or multipoint dissolution data comparisons are currently recommended.

According to the type of raw data (cumulative or non cumulative data set), the parameters determined will be different. The complete characterization of a profile, with amodel based on the theory of dissolution or semi empirical models, or with parameters estimated directly from raw data, provides different understanding of the in vitro behavior of the formulation.

Comparisons of cumulative dissolution profiles are performed in order to assess whether or not a drug product complies with preset specifications or to study the performance of various products under various experimental conditions. Dissolution profiles may be considered similar by virtue of overall profile similarity and similarity at every dissolution sample time point. Food and Drug Administration and Center for Evaluation and Research have collaborated to develop a specific test based on a model independent comparison.

In vitro in vivo correlation

In vitro in vivo correlation (IVIVC) has been defined as the establishment of a relationship between a biological property produced by a dosage form and a physicochemical characteristic of the same dosage form. For ER formulations, the dissolution test can serve as an indicator of how well the formulation will perform in vivo.

For pharmaceutical development, especially of extended release formulations, the relation between in vitro drug release and the biopharmaceutical performance in vivo needs to be confirmed as a valid and therapeutically relevant acceptance criteria. The application of in vitro dissolution tests to the control of critical production parameters, without establishing their relation within in vivo data would represent a misunderstanding in biopharmaceuticals. The determination of specification limits requires in vitro in vivo studies and comparison, and the establishment of a real and validated in vitro in vivo correlation. Provided that a valid in vivo in vitro correlation has been established, in vitro testing may be used in place of in vivo bioequivalence testing in the approval process for certain post approval changes in manufacturing and formulations. Three level of correlation (level A, B and C) have been defined by the USP and guidelines have been published from the consensus of academia, regulators and industry that build the foundation of the methodology.

To date, no universal dissolution test exists that in every instance gives the same rank order for in vitro dissolution and in vivo availability for different formulations or batches of the same drug. In vitro dissolution must be studied with different methods and devices in order to establish a validated in vitro in vivo correlation.

Conclusion

In vitro dissolution is perhaps the most important test method when developing a new dosage form. It is a very cost effective tool in the development of drug dosage forms. Dissolution testing remains a potentially powerful method for obtaining data related to quality, and to clinical performance of dosage forms, especially solid oral dosage forms. Well designed and accurately performed tests contribute to reducing development times and the number of in vivo experiment. If dissolution testing is not a surrogate for bioequivalence, the strength of the relation between dissolution and bioequivalence, that is the ability of a dissolution testing to predict the in vivo performance is very important.

RECENT ADVANCES IN DRUG ANALYSIS

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The chemical determination of drugs in its many different chemical forms and in complex matrices usually demands the use of proper analytical tools in order to achieve the ultimate goal. Frequently this goal includes the rapid determination of a main active substance together with its by products, metabolites and contaminants using modern instrumental techniques. This procedure, such as the determination of a drug in plasma starts with the sample preparation step, aiming the isolation of the target compounds from the matrix, followed in many cases by a clean-up step to isolate most undesirable interference's such as matrix constituents and finally the determination of the target analytes which usually includes a separation technique such as a chromatography coupled to an spectroscopic technique such as mass spectrometry.

In the last 10 years this picture has been changed with the development of new instrumental techniques that improves the speed, quality and efficiency of the available analytical methodologies. In addition to several improvements on the existing extraction techniques (such as miniaturization of liquid-liquid extraction and the automation of solid phase extraction) several other options where introduced for sample preparation in drug analysis including Supercritical Fluid Extraction (SFE), Accelerated Solvent Extraction (ASE), Solid Phase Micro Extraction (SPME), and Stir Bar Sorptive Extraction (SBSE), among others. The clean-up step has also been much improved with the introduction of a larger number of new phases for SPE as well as its automation using robotic systems that allows the unattended analysis of a large number of sample per day. SPME and SBSE are new techniques, which eliminates the use of solvents during the extraction step as well as the clean up procedure thus including all these steps in just one. After the extraction and clean-up steps (when required) the extract containing the target compounds has to be analyzed to determine its chemical nature (qualitative analysis) and amount (quantitative analysis); most analytical methodologies uses a chromatographic step to achieve this goal. Although capillary (high resolution) gas chromatography (CGC or HRGC) received a large attention as a major technique for drug analysis during several years in the recent past, its use has been replaced by other techniques such as high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC) and electrodriven separation techniques such as capillary electrophoresis (CE) and capillary electrochromatography (CEC). These techniques has also been on-line coupled with extraction techniques thus providing very powerful analytical techniques such as SFE-CE; SPME-HPLC; SPE-SFE-HRGC, and so on. The major advantage of these approaches is that the extraction, clean up and determination steps may all be computer controlled and performed on-line so that the sample will not be handled during the whole analytical procedure. Even using on-line coupled systems and highly selective detectors such as scanning fluorescence, laser-induced fluorescence, electrochemical detectors, and with the potential to obtain on-line full spectra of the target drug such us using PDA-based detectors, in many case a positive identification of the analyte is still mandatory. In such cases, the coupling of those separation techniques with mass spectrometry (MS) will provide the required identification tool. MS has been successfully coupled to gas chromatography (GC-MS), supercritical fluid chromatography (SFC-MS), liquid chromatography (LC-MS) and electrophoresis (CE-MS). Several types of MS analyzers are now commercially available to be used in those couplings including the more traditional quadrupoles, ion traps, time-of-flight, and other analyzers. In addition, the use of more than one analyzer in the same system has rendered the so-called tandem systems being the triple quadrupoles among the most popular ones. The goal of this lecture will be to present and discuss the most recent instrumental tool advances available for the analytical determination of drugs. The major advantages and limitations of each technique will also be addressed and several practical examples will be presented and discussed.

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VALIDITY, EFFICIENCY AND ETHICS IN BIOLOGICAL ASSAYS FOR DRUG DEVELOPMENT AND FOR QUALITY CONTROL

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Laboratory animals are used mainly as row materials for the production of immunobiologicals, as models or reagents for the development and testing of drugs, food additives and medical devises and as models for basic and research with the aim for extrapolation of data to humans or other animals. In order to assure reproducible animal experiments they must be performed by appropriate methods using laboratory animals with defined dramatype and controlled by genetic and microbiological monitoring programs. Reputable international and national regulatory agencies have established Good Laboratory Practices (GLP). Regulations intended to assure the quality and integrity of safety data collected on drugs, food additives and medical devises. They contain sections on all features of toxicology laboratory operation including the care and use of laboratory animals. In general, standard operating procedures must be outlined and rigorously followed and supported with detailed records. The breeding and care of laboratory animals has become a Science in itself leading to developing new experimental animals from domestic and wild animals, from mutants, from toxicology and by developmental and genetic engineering and assure their quality for the proposed use and the application of ethical principles to their care and use. Great strides have been made in standardising laboratory animals rised by intenational large breeders but the ever increasing difficulty in financing academic institution in the developing countries is hindering such improvement there.

The planning and design of a biological assays can vary greatly depending upon the objectives of the study and the availability of different types of experimental animals and equipment. According to ethical principles it is essential that the questions to be answered should be important, previously unanswered and of potential value for mankind. Refinement, Reduction and Replacement (The 3 Rs) were the general guiding principles since they have been introduced by Russell W.M.S. and Burch R.L. (1959)"The Principles of Human Experimental Technique", London, Methuen. Reduction should be understood as a need for fewer animals per project, refinement as a improvement of the method in order to reduce the distress to the animals and replacement means to change from the animal test another method or from conscious living vertebrates to non material. A well designed experiment should be unbiased, should have high precision, should make efficient use of resources, shoud have a wide range of applicability, should be of the appropriate size and correctly analysed and there should be a mean of quantifying uncertainty. A key point is to control the variability of the experimental material. Examples of factors that can introduce biers on increases random variation are: species suitability, genetic background, Sex, age, weight, microbial contaminations, diseases, cage size and design, population of animals per cage, poor handling and transportation, diet and water quality, dietary restrictions, sanitation treatment, pest control materials, noise, temperature, humidity, air exchanges, light intensity and periods, air quality, type and quality of bedding, mathod of euthanasia, experimental technique, dose range etc. In most scientific journals experimental animals are described poorly. A detailed animal definition is imperative for good science. The protocol might call for the use of imbred animals devoided of genetic variability but the heterogeneity found in outbreed stocks might be better for some studies.Experimental designs involving the use of randomized blocks, Latin squares, crossover designs and factorial arrangement of tratments are important tools for increasing the experimental efficiency. In fact studies shoud be designed to control every know source of variation with the exception of the sources beige studied. In many experiments published in the biomedical literature sample sizes were selected irrationally. The number of animals to be used depends on: effect size or Biologically/ Clinically significant diferrence, variability in the data, nature of the data and the statistical test to be use, significance level alpha (type I error), Power (1 -Beta) where Beta is the type II error, alternative hypothesis and expected lost animals during the study. Statistical significance and practical importance are not equivalent concepts. With massive numbers of animals it is sometimes possible to show that a very small difference is statistically significant. Similarly, using a very limited number of animals, it might not be possible to show that a clinically meaningful difference is statistically significant and a worthwhile effect might be missed. It is unethical to use either too many or too few animals in an experiment. An appropriate sample size should be estimated using power analysis or the resource equation method. For power analysis computer programs such as nQuery Adviser are available to do the calculations. The resource equation is only applicable to quantitative data. Experiments should allow performing statistical analysis including validity test, confidence limits and probability of errors. Statistics are the basis extrapolating from a small sample to the target population. IT is very important to involve a biostatidtician in the initial planning stage. Like other professionals, statisticians are usually most experiment in only a few areas of their discipline and many are unfamiliar with the types of biases and variability associated with animal research and advise less than optimally efficient methods of design and analysis. Survevs of published papers by a number of authors have shown that many experiments are poorly designed an/or analysed and that about a third of the experiments may be unnecessarily large. Most research workers appear to have a poor education in statistics and experimental design. This limitation must be overcome because legal and ethical considerations mandates that the evaluation of proposals to conduct activities involving animals include consideration of the number of animals to be used. The goal should be to derive the maximum amount of information form the minimum number of animals.

AM-01 POLYSACCHARIDE-BASED PHASE IN POLAR ORGANIC ELUTION MODE IN THE DETERMINATION OF ENANTIOMERIC PURITY OF A POTENT ANTI-HIV ANALOGUE NUCLEOSIDE

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Introduction

Racemic cis-2’, 3’-dideoxy-5-fluoro-3’-thiacytidine (FTC), Figure 1, is a new synthetic nucleoside that is unusually potent and selective against anti-human immunodeficiency virus (HIV) type 1 and 2 and hepatitis B *in vitro*. FTC was proposed by the manufacturer as racemic mixture, nevertheless it was recently demonstrated that the β-L (-) enantiomer of FTC is approximately 20-fold more potent against HIV h1 in peripheral blood mononuclear cells than is the β-D (+) enantiomer [1]. Therefore, the development of analytical methods that can separate and quantify these enantiomers plays a very important role in the drug development process.

Among the methods currently used to achieve chiral separation of racemic mixtures, liquid chromatography on chiral stationary phases (CSPs) has so far proven to be most useful method for this purpose. Numerous CSPs have been investigated and developed during the last decade, cellulose and amylose-based phases have been identified as very versatiles and useful chiral sorbents for the separation of enantiomers.[2]

We report here the direct enantiomeric resolution of FTC by HPLC on an amylose-based phase. A rapid and specific method was developed and validated for the determination of enantiomeric purity of β-(-)FTC and β-(+)FTC.

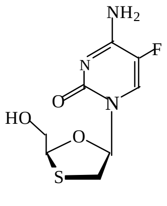

Figure 1- Chemical structure of FTC.

Objective

The purpose of this study was to develop a method for the determination of enantiomeric purity of β-(-)FTC and β-(+)FTC.

Materials and Methods

- **Chemicals**

Racemic FTC and its enantiomers were supplied by Microbiológica Química e Farmacêutica Ltda (Rio de Janeiro, Brazil). Methanol and acetonitrile (HPLC grade) were obtained from Mallinckrodt Baker (St. Louis, Missouri, USA) and other analytical grade reagents were obtained from Merck (Darmstadt, Germany).

- **Preparation of solutions and standards**

Two solutions 1mg.mL^{-1} and 5μg.ml^{-1} of both β-(-) and β-(+) FTC were prepared separately in the mobile phase for the preparation of calibration standards and for the quality control samples (QCs).

- **Instrumentation and HPLC conditions**

The HPLC system used consisted of one Shimadzu LC-10ATvp pump, an automatic injector SIL-10ADvp, a SPD-10Avp UV–VIS detector equipped with a SCL-10Avp interface. Data acquisition was performed using a Shimadzu CLASS-VP software.

The enantioseparation was performed on a column of amylose tris[(S)-1-phenylethyl-carbamate] coated onto APS-Nucleosil (7 μm particle size, 500 Å pore size, 20% w/w, 15 x 0.46 cm ID), prepared as described elsewhere [3]. Elution was carried out isocratically at a flow rate of 0.5 mL.min^{-1} with a mobile phase of acetonitrile:methanol (95:05 v/v) with 0.02% (v/v) trietylamine and 0.02% (v/v) of glacial acetic acid and monitored at λ=280nm.

- **Standard curve**

Using the appropriate stock solutions of β-(-) and β-(+)FTC standard solutions were prepared at the concentrations: 0.2- 2.0μg.mL^{-1} of the β-(-)FTC in presence of 100μg.mL^{-1} of the β-(+)FTC. The same procedure was done for the standards of the β-(+)FTC in presence of 100μg.mL^{-1} of the β-(-)FTC. The standards were prepared in triplicate.

Calibration curves were constructed by plotting the peak area against the concentration of standard solutions of each enantiomer. The data were subjected to linear regression analysis and showed good linearity with a correlation coefficient of 0.9990 for β-(-)FTC and 0.9995 for β-(+)FTC.

- **Precision**

The precision was determined using three QCs at 0.5, 1.1 and 1.8 μg.mL^{-1}of one enantiomer in presence of 100μg.mL^{-1}of the other. The peak-area ratios of the QCs were used for evaluating the inter-day and intra-day variability. Five samples of each control were prepared and analyzed in three different days.

- **Limits of detection and quantification**

The limit of detection and quantification were calculated according to the ICH Guideline [4].

Results and discussion

An amylose tris [(S)-1-phenylethyl-carbamate] phase eluted in polar organic mode buffered with trietylamine acetate (TEAA) gave an excellent enantioselectivity for the FTC enantiomers with a selectivity factor (α) of 1.9 and a resolution (R_s) of 3.3.

The limit of detection and quantification were estimated to be ca of 0.06 and 0.20% for β-(-) and 0.07 and 0.20% for β-(+)FTC. The intra-assay precision (coefficient of variation) and accuracy (deviation from nominal values) were measured by simultaneously assessing the QCs. The results are presented in Table 1 and led to satisfactory intra- and inter-assay precision, with coeficient of variation ≤2% for both enantiomers. The accuracy varied from 96 to 102% for β-(-)FTC and from 100 to 107% for β-(+)FTC. The method developed was highly suitable for the determination of the enantiomeric purity of β-(-)FTC and was validated for the quantification of β-(+)FTC as an impurity in a range of 0.2-2.0%. A typical chromatogram is showed in Figure 2.

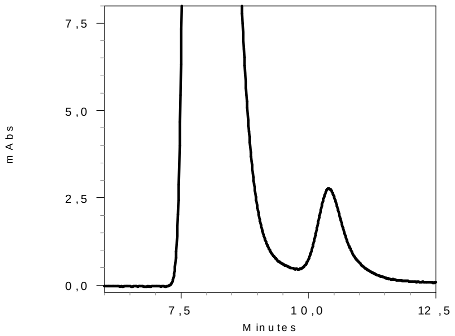

Figure 2. Chromatogram of β-(-)FTC spiked with 1% of β-(+) FTC.

Although, it is not the enantiomer of interest, the same procedure was validated for measuring the enantiomeric purity of β-(+) FTC.

Conclusion

A rapid, precise and accurate chiral HPLC method was developed and validated for the measurement of the enantiomers of FTC. The method is able to accurately determine 0.2% of each enantiomer, as an impurity. These limits meet the requirements of the pharmaceutical industry, so it can be used efficiently for assessing enantiomeric purity in bulk products.

Acknowledgments
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Table 1

Precision and accuracy data of β-(-)FTC and β-(+)FTC, intra-day (n=5)

	Concentration μg.mL^{-1}	1st day		2nd day		3rd day	
		CV(%)	Accuracy(%)	CV(%)	Accuracy(%)	CV(%)	Accuracy(%)
(-)FTC	0.5	1.03	102	1.87	100	1.88	102
	1.1	1.51	100	1.54	100	0.86	101
	1.8	0.62	96	0.47	96	0.65	96
(+)FTC	0.5	1.39	102	1.44	100	0.39	101
	1.1	0.88	107	1.79	106	1.52	103
	1.8	2.06	102	0.56	102	0.87	102

AM-02 EFFECTS ON ENANTIOSELECTIVITY BY THE USE ON POLYSACCHARIDE-BASED COLUMN ON MULTIMODAL ELUTION

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Introduction

Among the most useful and versatile chiral columns described are polysaccharide-based columns. Recently, it was demonstrated that those columns could be efficiently used on multimodal elution [1] widening the applicability of these chiral phases for the enantioresolution of variety of chiral compounds.

A complete study for the enantioresolution of omeprazole, lanzoprazole, pantoprazole and metyrapol using amylose tris(3,5-dimethoxyphenylcarbamate), tris[(S)-1-phenylethylcarbamate] and tris(3,5-dimethylphenylcarbamate) phases, on multimodal elution, was carried out. Great differences in enantioselectivity for each phase were observed on the different modes of elution for the series of benzimidazoles investigated and also for metyrapol. This work reports these results.

Experimental

- **Equipment and columns**

Two liquid chromatographic systems were used. One consisted of a Shimadzu LC-10AD pump, a Rheodyne 7125 injector fitted with a 50 µL loop, a SPD-6AV UV-VIS spectrophotometric detector and a C-R6A Chromatopac recorder. The other was a Shimadzu system with two LC-10ADvp pumps, a SDP-10Avp detector and an auto sampler SIL-10ADvp with a SCL-10Avp interface. In this system the data acquisition was performed using a CLASS-VP software.

The columns (150 x 4.6 mm ID) were prepared as described elsewhere [2,3] and consisted of amylose tris(3,5-dimethoxyphenylcarbamate) coated (20% w/w) onto APS-Nucleosil (7 µm particle size and 500 Å pore size), amylose tris[(S)-1-phenylethylcarbamate] coated (20% w/w) onto APS-Nucleosil (7 µm, 500 Å) and amylose tris(3,5-dimethylphenyl carbamate) coated (20% w/w) onto APS-Hypersil (5 µm, 120 Å).

- **Chemicals**

Racemic omeprazole, lansoprazole and pantoprazole were donated from Libbs Farmacêutica Ltda, Boehringer do Brasil and from Novartis Biociências S. A., respectively.

Metyrapone was purchased from Aldrich. Racemic metyrapol was synthesized by sodium borohydride reduction of metyrapone. The resulting alcohol was characterized by IR and ^{1}H-NMR analysis.

Solvents were either HPLC grade from Merck or from Mallinckrodt. Water was purified with a Milli-Q system (Millipore).

- **Solutions**

Stock solutions of (±)-omeprazole, (±)-lansoprazole and (±)-pantoprazole (100 µg/mL) were prepared in methanol and (±)-metyrapol solution (100 µg/mL) was prepared in ethanol.

Aliquots (20 µL) of the stock solutions were placed in a glass tube and the solvent evaporated under a stream of nitrogen. The dried analytes were reconstituted using 200 µL of mobile phase and the solutions were vortex-mixed for 15 s. A 50 µL aliquot was then injected onto the chromatographic system.

- **Chromatographic conditions**

The chromatographic analysis were performed at ambient temperature using the following mobile phases: hexane:2-propanol (60:40 v/v), hexane:ethanol (70:30 v/v), ethanol, methanol, acetonitrile, acetonitrile:methanol (95:05 v/v), acetonitrile:methanol (90:10 v/v), acetonitrile:water (50:50 v/v), acetonitrile:water (30:70 v/v) and methanol:water (70:30 v/v). The flow-rate was 0.5 or 1.0 mL/min. The dead times (to) were determined using 1,3,5-tri-*tert*-butylbenzene for normal and polar organic elution and acetonitrile for reversed phase mode. Omeprazole (**1**) was detected at λ= 302 nm, lansoprazole (**2**) and pantoprazole (**3**) at λ= 285 nm and metyrapol (**4**) at λ= 260 nm (Figure 1).

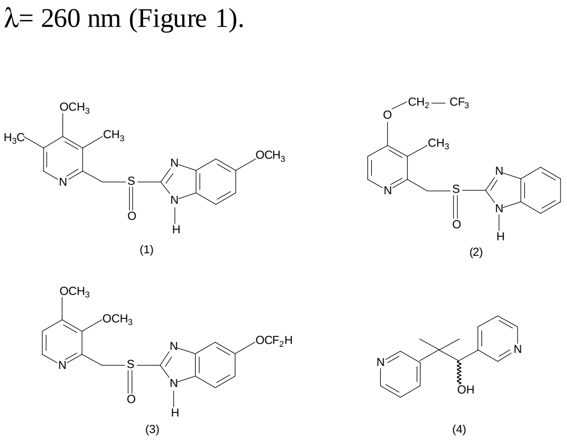

Figure 1: Selected chiral compounds

Results and Discussion

Knowing that different selectivity could be gained by altering the elution mode [4}, the aim of this work was to investigate the effects in this changing on the enantioselectivity of the polysaccharide-based phases. For doing this investigation, the benzimidazoles series of compounds: omeprazole, lanzoprazole and pantoprazole were selected with metyrapol. The reason for selecting these compounds is justified since we are currently developing assays for analyzing them in biofluids by direct injection and the use of reversed phase elution is recommended since it makes the coupling of columns a much easier task.

The amyloses chiral phases were selected based on previous work for the enantioresolution of a series of chiral sulfoxides and also of some alcohol [2-3,5].

Various changes in mobile phase composition were examined on each chiral stationary phase to determine the effects of the organic modifier on the resolution of the chiral compounds. The retention (k), selectivity factor (α) and resolution (Rs) were examined for these selected compounds in each mode of elution at the same chromatographic conditions. For some individual compounds small alterations on the organic modifier were made in order to improve resolution.

The amylose tris(3,5-dimethoxy-phenylcarbamate) phase showed the highest enantioselectivity for the series of drugs examined. Lansoprazole and pantoprazole were enantioresolved with excellent selectivity and resolution on reversed phase mode using ACN:H_2O (50:50 v/v) as mobile phase, while metyrapol had partial resolution. When the solvent strength was decreased to (30:70 v/v) an increase in selectivity was observed for the metyrapol enantiomers and good resolution was obtained (Rs=1.84). Omeprazole could not be resolved on this mode of elution, even when methanol was used as the modifier. However, when methanol was used as the eluent on the polar organic mode, the enantiomers of omeprazole could be separated with excellent selectivity and resolution. Lansoprazole showed good selectivity but poor resolution while pantoprazole had good selectivity and resolution. None of the compounds could be enantioseparated on normal elution mode using this chiral phase.

With the amylose tris[(S)-1-phenylethylcarbamate] phase the separation factor and the resolution obtained were excellent for omeprazole, lansoprazole and pantoprazole only on normal phase condition using hexane:ethanol (70:30 v/v) as mobile phase, while metyrapol was not resolved on any condition examined.

The amylose tris(3,5-dimethylphenyl carbamate) phase showed excellent selectivity for omeprazole on normal and organic polar elution mode using hexane:ethanol (70:30 v/v) and methanol respectively as mobile phases. No resolutions or very poor ones where obtained for lansoprazole and pantoprazole on the different modes of elution examined with this chiral phase.

Metyrapol was enantioseparated in this phase on the three elution modes. When ACN:MeOH (95:05 v/v) was used as mobile phase a separation factor of 1.32 and resolution of 1.21 was observed. Increasing the methanol content to 10%, made no effect on the selectivity while the resolution was slightly increased (Rs =1.45). This chiral phase showed the best enantioselectivity (α = 1.85) for this compound on reversed phase conditions using ACN:H_2O (50:50 v/v) as the mobile phase.

Conclusion

The systematic comparative study of the three chiral phases on the three different modes of elution for the four compounds examined has highlighted that the enantioselectivity of these polysaccharide-based columns can be dramatically varied by changing the type and composition of the mobile phase. The retention and separation factors and also the resolution can be significantly increased and optimized by modifying the mobile phase.

Acknowledgments

FAPESP and CNPq.

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AM-03 HPMA AND HEMA PACKING MATERIALS FOR APPLICATION IN AQUEOUS SIZE EXCLUSION CHROMATOGRAPHY (SEC)

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Introduction

Organic porous hydrophobic polymer sorbents, for example, polystyrene based materials, and hydrophilic sorbents derived from dextran have been widely used in size exclusion chromatography (SEC) for the purification and reverse phase separation of macromolecules. However, a disadvantage to organic based sorbents is the need for organic solvents and the resultant difficulty in solubilizing hydrophilic materials. On the other hand, hydrophilic sorbents are often not able to withstand high flow rates.

Objective

To produce synthetic hydrophilic polymers which can overcome these problems, because:

∗ They can be used with hydrophilic solvents
∗ They are more resistant to pressure and
∗ The exclusion volume and hydrophilicity can be controlled by careful use of porogen and monomers.

Methodology

Two different porous packing materials were synthesized by suspension polymerization using hydroxypropyl methacrylate (HPMA) and hydroxyethyl methacrylate (HEMA) monomers, cross-linked with ethyleneglycol dimethacrylate (EDMA) in the presence of dodecanol (DOD) and initiator 2,2, azobis-2-methylpropionitrile (AIBN). The suspending media used were polyvinyl alcohol and magnesium hydroxide, prepared in situ with different agitation speeds at 70°C. Scanning Electron Microscopy (SEM), Nitrogen Sorption Analysis (NSA) and Mercury Intrusion Porosimetry (MIP) were used to characterize the porous beads. Column packings were prepared in glass and stainless steel columns using low and medium pressure pumps, respectively, and tested for the separation of model macromolecules (polysaccharides and proteins).

Results and Discussion

The beads formed a fine, loose white powder, which could be readily packed by a slurry suspension. SEM was used to evaluate the beads morphology and the results revealed the formation of porous HPMA (Figure 1) and HEMA beads (Figure 2).

The NSA and MIP results [specific surface area/SSA (m^2g^{-1}); total pore volume/TPV ($x10^{-3}$ mLg^{-1}); average pore radius/APR (Å); maximum pore size/MPS (Å)] measured for HPMA and HEMA respectively were consistent with the SEM results indicating a high degree of porosity directly proportional to the amount of the porogen, dodecanol, used during the suspension polymerization process. There appears to be an optimal amount of porogen to produce the best shape, texture and porosity profiles in the selected batches of porous beads produced (Table 1).

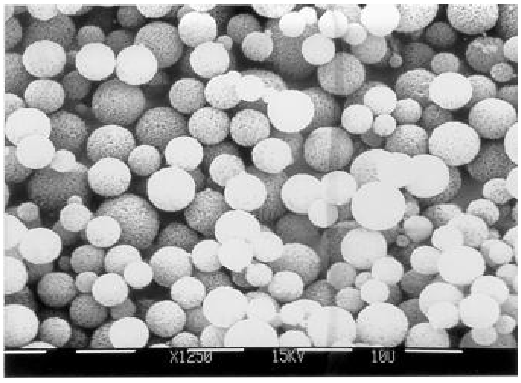

Figure 1. SEM photograph of HPMA beads obtained with 41.25% addition of porogen (X1,250, scale bar 10 μm)

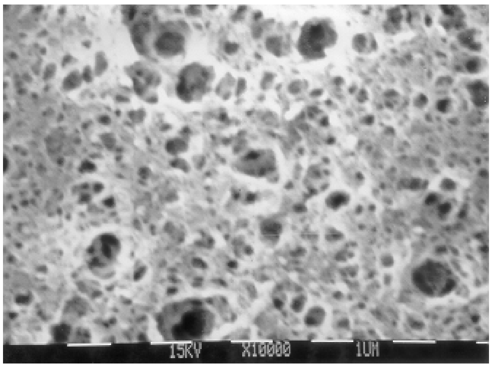

Figure 2. SEM photograph of HEMA beads obtained with 20% addition of porogen (X10,000, scale bar 1 μm)

Table 1. NSA and MIP results for HPMA and HEMA beads

Polymer	SSA (m^2g^{-1})	TPV ($10^{-3}mLg^{-1}$)	APR (Å)	MPS (Å)
HPMA Beads	142.0	350.7	49	583
HEMA Beads	31.2	73.9	441	2,400

The packed columns showed a linear range of macromolecule separation after calibration with polysaccharides reference standards, dextrans (MW 2,000,000-10,000) of narrow molecular weight distribution.

In the application of the designed chromatographic packing material, polysaccharides injected in the HPMA packed stainless steel column (Figure 3) and in the HEMA packed glass column (Figure 4) could be separated according to size exclusion upon proper adjustments of mobile phase.

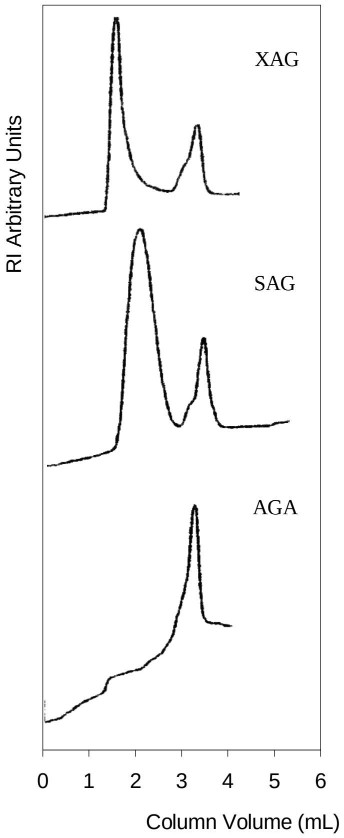

Figure 3. Representative chromatograms of polysaccharides, Xanthan Gum/XAG, Sodium Alginate/SAG and Agarose/AGA aqueous solution injected in the HPMA packed stainless steel column (300 x 4.6 mm), using 0.1% w/v aqueous sodium azide as mobile phase, 0.3 mL/min flow rate, RI detector and 100 mV register attenuation.

Conclusion

The successful preparation of the resins indicate that HPMA and HEMA beads can separate a wide range of high molecular weight compounds including polysaccharides and proteins (results not shown) through the size exclusion mechanism.

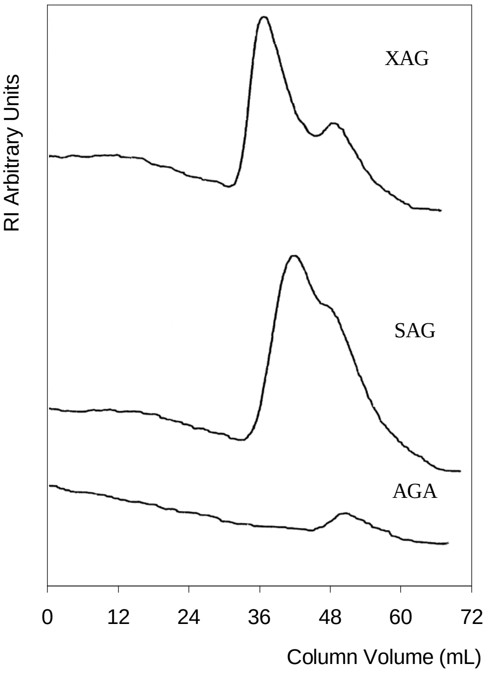

Figure 4. Representative chromatograms of polysaccharides XAG, SAG and AGA aqueous solution injected in the HEMA packed glass columns (50 x 1.5 cm), using 0.1% w/v aqueous sodium azide as mobile phase, 1.5 mL/min flow rate, RI detector and 10 mV register attenuation

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AM-04 THE PIRKLE TYPE α-BURKE 2 CHIRAL STATIONARY PHASE: ENANTIOMERIC SEPARATION AND RETENTION BEHAVIOR OF FEW β-BLOCKERS

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Introduction

Propranolol, atenolol and metoprolol are predominantly used in angina pectoris, in certain arrhythmia's and in systemic hypertension [1-5]. Majority of the β-blockers and many synthetic drugs are marketed as racemic mixtures, despite the fact that enantiomers may show different pharmacodynamic and pharmacokinetic profile [3, 5, 6]. Over the last decade, many analytical and preparative high performance liquid chromatographic columns have been developed and commercialized for rapid enantiomeric separation of chemical compounds [3]. These columns are often referred as chiral stationary phase (CSPs). The α-Burke 2 is a new brush or Pirkle CSP a π-acceptor CSP, specially developed to separate beta-blocker enantiomers [3, 6]. The elution order of enantiomers is predictable since the sterio-chemistry of the CSP is known. However, the high chiral recognition ability of this column has been exploited to a limited extent. We started this work with an objective to study and exploit the factors effecting enantio-separation, such as column operating temperature, polarity, and organic modifier in the mobile phase on selectivity and efficiency of the column. These parameters are being exploited in order to select chromatographic conditions, which lead to maximize chiral selectivity and appropriate relative retention (k) so that the method could be applied at analytical and preparative scale.

Experimental

Materials and Method

- **Reagents and solutions**

Racemic AT, PR and racemic MT (+) tartrate salt were purchased from Sigma (St. Louis, MO, USA). R-PR, S-PR, R-AT and S-AT (99%) were purchased from Aldrich (Milwaukee, WI, USA). AstraZeneca R&D, Mölndal, Sweden kindly donated the R-MT hydrochloride (H 150/65) and S-MT hydrochloride (H 150/64). The enantiomeric purity of R- and S-MT was found to be 98%. All the solvents were of HPLC grade except ammonium acetate (analytical grade). Dichloromethane, acetonitrile, methanol, ethanol and isopropanol were obtained from Merck (Darmstadt, Germany).

- **Instrumentation**

Chromatography was performed using either of two systems. 1) HPLC system, model 5000 (Varian Associates, CA, USA); rotatory injection valve, Model 7125, Rheodyne® with a loop of 20 μL; variable ultraviolet detector, model Varian 4000; integrator model Varian 4400, (Varian Associates, CA, USA); 2) HPLC system consisting of a Shimadzu LC-10ADVP pump, (Shimadzu Corporation, Japan), a auto-injector equipped with a 50 μL loop SIL-10ADVP (Shimadzu Corporation, Japan), a UV/VS photodiode Array detector SPD-M10AVP (Shimadzu Corporation, Japan), a column oven CTO-10ASVP (Shimadzu Corporation, Japan). Chiral stationary phase: α-Burke 2® (250 x 4,6 mm i.d.) (dimethyl N-3, 5-dinitro-benzoyl-α-amino-2,2-dimethyl-4-pentenyl phosphonate covalentely bonded to 5μm silica) (Regis Technologies, Inc. Morton Grove, IL).

- **Analytical conditions**

Analytical conditions for AT, PR and MT were standardized through high performance liquid chromatography system using α-Burke 2® as chiral stationary phase. The samples were chromatographed at room temperature (24°C ± 2), except to study temperature effect, with an injection volume of 20 μL, a flow rate of 1.0 mL/min, ultraviolet detection was made at 276 nm. The mobile phase was constituted by a mixture of dichloromethane or acetonitrile modified with methanol, ethanol or isopropanol along with ammonium acetate. The mobile phases were prepared fresh each day and vacuum filtered through hydrophobic membrane, 0.22μm, Millipore® GVHP, and degassed for 20 minutes.

Results

It was observed that increase in eluent polarity, either concentration or type leads to decreases in capacity factor value (k_2) of propranolol (PR), metoprolol (MT), and atenolol (AT). Peak broadening and increase in k_2 value were observed beyond critical eluent polarity. The enantio-selectivity was greatly affected by polarity and nature of eluent. The critical ammonium acetate concentration was established at 12 mM (Table 1). Acetonitrile showed better enantio-separation as compared to dichloromethane. Methanol in low concentration (20%) improves peak shape and detection without compromising enantio-separation (Fig. 1). The effect of column operating temperature on enantio-separation will be discussed.

Table 1.
The effect of amm. ac. on k_2 and α of PR, MT & AT enantiomer (Conditions: DCM:ethanol (80:20), 1.0mL/min. 24°C ± 2).

amm. ac. (mM)	k_2			α		
	PR	MT	AT	PR	MT	AT
4	13.53	8.18	-	1.29	1.12	-
8	5.21	3.09	9.18	1.27	1.11	1.07
12	3.19	2.11	6.42	1.27	1.15	1.06
16	2.44	1.40	4.69	1.27	1.11	1.05
20	1.91	1.07	3.61	1.27	1.10	1.04
24	1.67	0.95	3.00	1.27	1.10	1.05
28	1.44	0.80	2.61	1.27	1.11	1.04
32	105	0.64	2.24	1.28	1.10	1.04

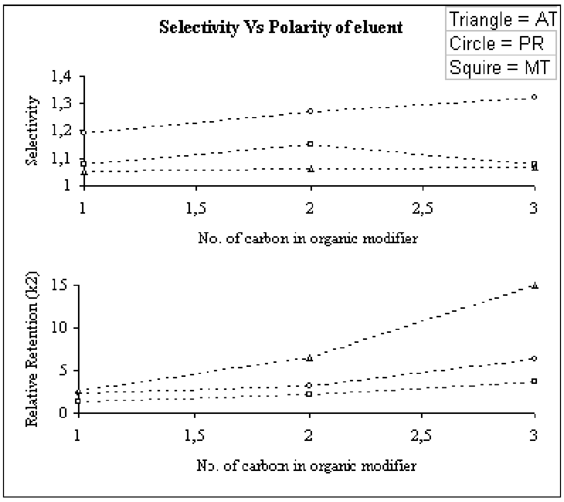

Figure 1. The effect of polar organic modifier on selectivity and relative retention of more retained enantiomer (Conditions: DCM:organic modifier: amm. acetate 12 mM, 1.0mL/min. 24°C ± 2)

Conclusions

Enantiomeric separation of PR, MT and AT was obtained without derivatization and the methods can be automatized for quantitative routine analysis. The concentration of the polar organic modifier (ethanol, methanol and isopropanol), the concentration of protonizing agent (amm. ac.) in the eluent and operating temperature greatly influence the selectivity, peak shape and relative retention of enantiomers. Operating temperature or organic modifier did not affect elution order.

Acknowledgements

We are thankful to FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for financial assistance and to Dr. Paul K. Owens, AstraZeneca R&D, Mölndal, Sweden for providing R-MT & S-MT standards

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AM-05 CONTINUOUS CHROMATOGRAPHIC SYSTEM FOR SEPARATION OF PHARMACEUTICAL PRODUCTS BASED ON CHIRAL RECOGNITION

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Introduction

A general theme in the field of separation processes is the generation of improved selectivity among solutes. The power of modern synthetic chemistry, in conjunction with separation science and technology can be used to develop new separating agents and equipments with enhanced functionalities for selective separation. There is a growing demand in the pharmaceutical industry for efficient and cost effective methods to purify optical isomers, and the use of continuous chromatographic processes has been pointed out as an important tool to match the objectives of chemical manufacturing of several chiral compounds [3]. Simulated moving beds (SMB) is a large-scale version of the traditional high-performance liquid chromatography (HPLC), but unlike normal HPLC, SMB is a continuous process in which a solvent and the compounds to be separated are injected into and withdrawn from a ring of chromatographic columns at rotating points between the columns. This version of continuous process has been used for several authors to separate components from racemic mixtures [2], obtaining the two enantiomers of chiral molecule with a high purity and convenient quantities to perform clinical tests or even production stages. The variety of chiral selectors used as stationary phase and the vast number of racemic mixtures produced in the pharmaceutical industry turns this technique a powerful tool, leading to a stimulating and challenge area of interest both for laboratory scale studies and production plant design. This work is related to the design, mounting and utilization of a laboratory-scale unit to separate racemic mixtures. The stationary phase utilized was cellulose-triacetate in a microcrystalline form, which has been considered one of the most convenient stationary phases for the resolution of racemates [1]. The aim of the present work was to design, mount and utilize a laboratory-scale unit to separate racemic mixtures of pharmaceutical compounds originated from organic synthetic routes. This unit is, by our current knowledge, the unique SMB chromatography research oriented and laboratory-scale unit being operated in Brazil. The main instrumentation of the unit is discussed and results are reported for the beds porosity.

Material and Methods

- **Simulated moving bed chromatography unit**

The laboratory scale SMB unit built has eight columns of 1.6 cm diameter and height of 15.0 cm. The adsorbent is maintained in the columns by appropriate holders of variable height position. Figure 1 is a general scheme of the unit, showing that the dessorbent is recycled outside of the cycle. This is performed by using a multi-position valve instead of a solvent reflux pump. Other four multi-position valves are responsible by the position change of feed, rafinate and extract withdraw at designed switch times. These valves are connected with four semi-preparative liquid chromatographic pumps (Shimadzu LC-6AD), having flow-rates up to 25 mL/min. In Figure 2 is shown some detail of the complete set up .Multi-position valves (Valco Instruments Co.) are electrically actuated and linked to a computer by a data-acquisition plate. The control of each valve is done by a software program on an automatic way, to perform operation of the unit at the chosen flow-rates parameters. The total amount of analyte in rafinate and extract. is determined through an UV/VIS detector equipped with flow-cell. The difference of those concentrations can be determined by the use of a polarimetric detector also equipped with a flow cell.

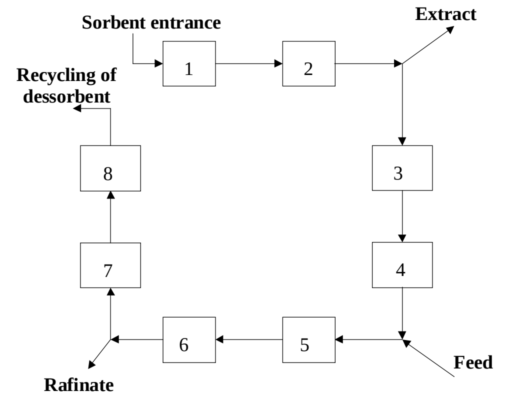

Figure 1: Basic scheme of the SMB laboratory-scale unit

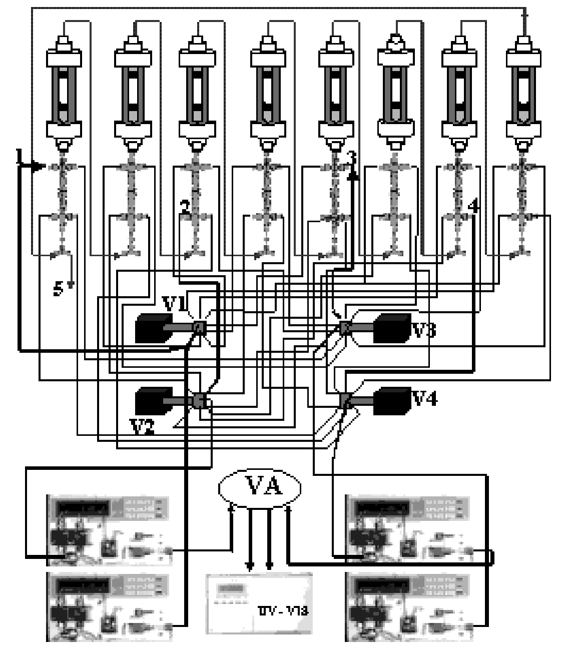

Figure 2: General set-up of the SMB unit

Results

Bed porosity is an important parameter for the unit design. Measurements of MCTA packed beds porosities were done following the protocol disclosed by Nicoud (1993), using the injection of 1,3,5 - Tri - tert – butilbenzene. This compound does not react with the adsorbent and so the residence time through the bed is proportional to the bed porosity.

A pulse injection using the inert compound results on a peak detected by an UV device results on the determination of the residence time . A typical experimental result for the residence time is depicted in Figure 3. This result was obtained with the 1,3,5 tert-butyl benzene and microcristaline triacetate as a stationary phase packed in Superformance columns (Merck) of 1.6 cm diameter and 15 cm length. Typical residence times for this set up are around 11 minutes and the resultant bed porosity is 0.480.

Conclusion

The method for porosity determination is reliable, leading out to accurate results of this variable. The SMB unit was mounted from separate equipments parts and has as main advantage in a next future, the flexibility of obtaining experimental data for continuous chromatography of binary systems on an appropriate scale aiming at the scale up of an economically competitive HPLC process.

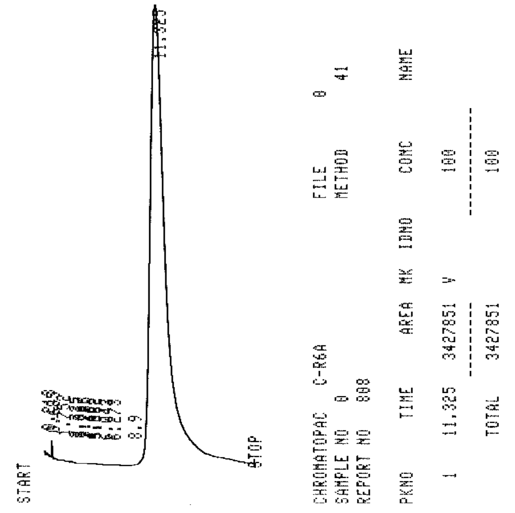

Figure 3 – Peak obtained with the injection of 1,3,5 - Tri - tert – butilbenzene in a column packed with MCTA. Flow rate is 1mL/min.

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AM-06 ALTERNATIVE METHODS TO ANALYSE CAPTOPRIL IN TABLETS

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Introduction

Captopril, an angiotensin-converting enzyme inhibitor, synthesized by Ondetti and Cushman in 1977 [1], is one of the most used therapeutic agents to control hypertension. It is commercialized in Brazil as tablets containing 12.5, 25 or 50 mg of captopril. The monograph of captopril tablets is included in the last editions of the United States [2] and British [3] Pharmacopoeias. Both specify very similar reverse phase liquid chromatography methods to analyze captopril in tablets using UV detection at 220 nm. Nevertheless, titrimetric methods using potassium iodate 0.1 N [2] or iodine 0.05 M [3] are applied to analyze captopril raw material. In addition, an ultraviolet spectrometric method is used to determine captopril in the dissolution test [2].

Objective

The aim of this work was to present alternative simple methods to analyze captopril in tablets, as a contribution to the Brazilian Pharmacopoeia [4] captopril tablets monograph. A comparison was carried out between the official USP 24 method with a spectrophotometric and a titrimetric methods.

Methodology

Tablets containing 25 mg of captopril and its respective placebo were analyzed by liquid chromatography, ultraviolet spectrophotometry and titration method.

The liquid chromatography was performed on a LC 10-AS Shimadzu chromatograph, with a SPD 10-AV Shimadzu ultraviolet detector, using the conditions described in the USP 24 captopril tablets monograph, to determine captopril and captopril disulfide.

For the ultraviolet spectrophotometric method, a quantity of the powdered tablets corresponding to 50 mg of captopril was transferred to a 100 mL volumetric flask with a mixture of ethanol/water (1:1). The preparation was sonicated for 30 minutes, diluted to the volume, shaken and filtered. The solution was diluted to a 0.002% w/v with the same solvent. A standard solution was prepared at the same conditions. The absorbances were determined at 212 nm, in a UV-160A Shimadzu spectrophotometer.

The titrimetric method was performed by transferring a quantity of the powdered tablets equivalent to 300 mg of captopril to a glass-stoppered flask and adding 100 mL of water. The flask was sonicated for 10 minutes and shaken for more 15 minutes. There after, 10 mL of 10% v/v sulfuric acid, 1 g of potassium iodide and 2 mL of starch solution were added. The titration was accomplished with potassium iodate 0.015 M to a faint blue end-point, which persisted for 30 seconds. A blank was also performed. Each mL of potassium iodate is equivalent to 19.56 mg of captopril.

Results and Discussion

Both captopril and captopril disulfide showed good resolution (R = 3.96) in the chromatogram of the resolution solution (Figure 1). The concentration found for captopril disulfide in the tablets was 0.3%, below the maximum limit of 3.0% [2]. The assay result for captopril was 100.56% of the labeled amount. The results presented good intra-day (RSD = 0.23%; n= 6) and inter-day precision (RSD = 1.09%; three days; n=22). The assay chromatogram did not present other different peaks. This indicates no interference from excipients at 220 nm.

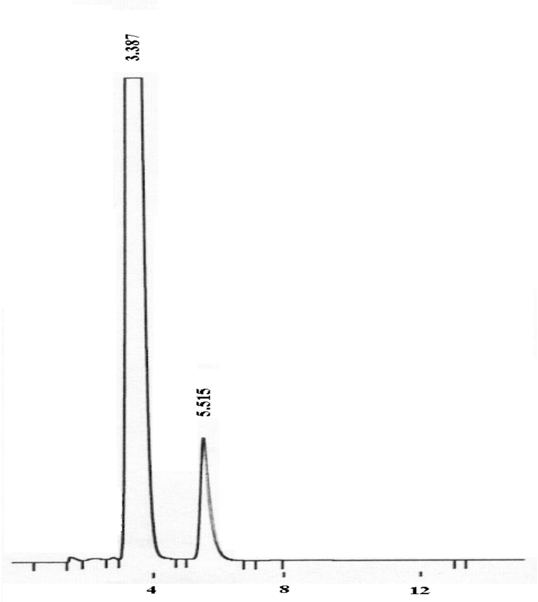

Figure 1- Chromatogram of a resolution solution of captopril 1 mg/mL (3.387 min) and captopril disulfide 0.03 mg/mL (5.515 min), R = 3.96. Conditions: USP 24 [2].

Both captopril and captopril disulfide showed identical ultraviolet spectra, with maximum about 205 nm. The absorbances found for a 0.002 % w/v captopril and captopril disulfide standard solutions were approximately 0.5 (Figure 2). The analysis was carried out at 212 nm as referred in captopril tablets dissolution test of USP 24 [2]. The absorbances measurements at 212 nm were very close to those obtained at 205 nm, wherein the selectivity is lower. The captopril content obtained by the spectrophotometric method was higher (101.84% of the labeled amount) than that obtained by HPLC method (100.56%). The intra-day method precision was also worse (RSD = 1.91%; n=6) than the HPLC method. The absorbance of the placebo solution was 0.013 at 212 nm. This indicates a little interference from the excipients. The higher results obtained, compared to HPLC results, can be explained both by the interferences of excipients and the presence of captopril disulfide (0.3%). These evidences claim that the spectrophotometric method is not a good option for the captopril analysis in tablets.

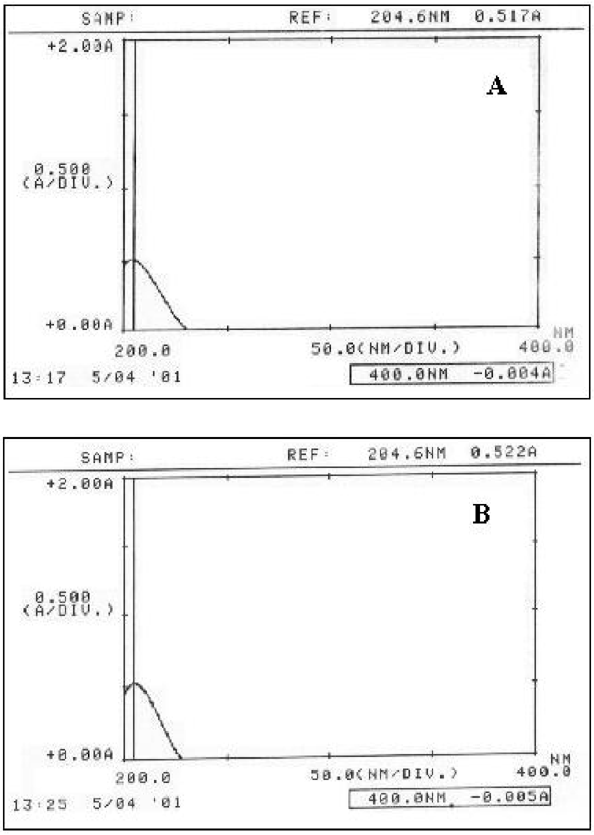

Figure 2 - Ultraviolet spectra of a 0.002 % w/v captopril (**A**) and captopril disulfide (**B**) standard solutions in ethanol/water (1:1).

The titrimetric method is based on the oxidation of captopril by potassium iodate producing the corresponding captopril disulfide. The titration of the corresponding amount of the placebo consumed less than 0.1 mL, showing that there was no interference from the excipients. These considerations could explain the fact that the found result (100.53% of the labeled amount) was more compatible with that obtained by HPLC method. In addition, the visualization of the end point was clear to faint blue color and the method precision was satisfactory (RSD = 0.49%; n=6).

Conclusion

The results suggest that the titrimetric method could be an alternative to determine captopril in tablets, as long as the degradation product (captopril disulfide) and the excipientes used in the formulation do not react with the potassium iodate in the established conditions for the titration.

Acknowledgments

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AM-07 A COMPARATIVE STUDY OF METHODOLOGIES FOR THE QUANTIFICATION OF CHLORHEXIDINE GLUCONATE IN AQUEOUS SOLUTIONS – PRELIMINARY ASSAYS

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Introduction

Chlorhexidine in aqueous solution has been largely indicated as an antiseptic in European countries. In Brazil we observe an increase in the number of preparations for external uses containing chlorhexidine gluconate and, more recently, its indication in odontology as an oral antiseptic [1].

Chemically, chlorhexidine is classified as a cationic bisbiguanide and it shows a wide range of applications, acting on Gram-positive and Gram-negative bacteria [2, 3].

Aqueous solutions of chlorhexidine gluconate 20% w/v are available in the local market and have been used to prepare diluted preparations.

Since the Brazilian Pharmacopoeia has not still included the monograph for this antiseptic, other pharmacopoeias were consulted to evaluate the analytical methods used.

The European [4] and Portuguese [5] Pharmacopoeias specify neutralization volumetry in non-aqueous media as a methodology for aqueous samples, with potentiometric determination for the end point of the titration. The Japanese Pharmacopoeia adopts the same methodology, but it recommends that the samples be previously reduced to a residue in a steam bath.

In this work the aim is to evaluate and compare the nonaqueous titration of the liquid and previously evaporated residual samples using both potentiometric and a color visual end point.

Experimental

- **Material**

E510 Methron Herisau potentiometer; Ingold double membrane conjugate electrode (glass/silver-silver chloride).

- **Reagents**

Glacial acetic acid PA (MERCK); acetic anhidride PA (REAGEN); perchloric acid (REAGEN) 0.1 mol/L, volumetric solution in acetic acid; crystal violet solution (MERCK) 0.1% (w/v), in acetic acid; chlorhexidine gluconate 20% (w/v), aqueous solution (EMELFAR).

Methods

2.0 mL-samples of the chlorhexidine gluconate solution were titrated in triplicate, containing 0.4 g equivalent of the salt. Liquid samples and previously residual samples were used.

Samples were added with 50 mL of glacial acetic acid and titrated with a volumetric solution of perchloric acid 0.1 mol/L. For the determination of the end point of the titration two methods were used: visual determination with a crystal violet solution, and potentiometric determination. The first derivative was applied in the potentiometric determination to aid the end point evaluation.

Results

Although chlorhexidine is unstable at high temperatures [7], its salt samples showed no sign of decomposition after evaporation in steam bath. It was not possible to visualize the end point for the potentiometric determination of non-treated samples, even with the application of the first derivative. On the other hand, the visualization was clear for the residual samples (Figure 1) and it coincided with the change in color of the indicator from violet to blue. Furthermore the precision of the analysis was greater for the residual samples, regarding samples without any treatment. Table 1 presents results of the quantitative determination of chlorhexidine gluconate in the different described conditions.

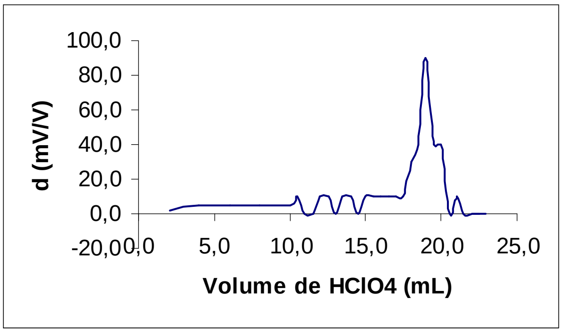

Figure 1 – First derivative of the titration curve of the residual sample of chlorhexidine gluconate, by titration with 0.1 mol/L perchloric acid in acetic acid.

Table 1 – Determination of the chlorhexidine gluconate content in 20% w/v aqueous solution by titration with perchloric acid 0.1 mol/L in acetic acid.

Condition	Content % (w/v)	Average % (w/v)	RSD (%)
Liquid sample, visual end point	22.07 23.30 23.21	22.86	3.00
Liquid sample, potentiometric end point	End point not visualized		
Residual sample, visual end point	18.83 18.96 19.20	19.00	1.01
Residual sample, potentiometric end point	19.79 19.67 19.79	19.75	0.32

Values of relative standard deviation (RSD) for the residual samples, analyzed by visual detection or potentiometrically, were significantly less than the values obtained for the liquid samples in the analysis with the color indicator.

The percent content results found with the color indicator assays of the liquid samples were low, since the results are well above those obtained for the dried samples. The specification limits lie between 19.0 and 21.0% of chlorhexidine gluconate.

Results suggest that the presence of water interferes in the precision as well as in the accuracy, and also hampers the end point visualization in the potentiometric determination.

Conclusion

Although pharmacopoeias assign the neutralization volumetry in non-aqueous technique as the most common method for the evaluation of chlorhexidine gluconate in aqueous solutions, with a potentiometric end point, the visual detection of the end point could be considered as an alternative, given the precision obtained for the results.

Data indicate that both the potentiometric and the color visual end points can be used for the analysis of chlorhexidine gluconate solutions, as long as the liquid samples are previously evaporated in a steam bath to residual samples.

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AM-08 HEAVY METALS DETERMINATION IN MEGLUMINE ANTIMONATE BY INSTRUMENTAL METHODS

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Introduction

Determination of inorganic impurities of drugs and medicines is a quite important subject in the quality control. A lot of trace elements are toxic and some of them even in very low concentrations could decrease the stability of active ingredients. In addition, some of the impurities could be indicators of non-adequate handing and storage or be used as fingerprints for the source of drug crude material [1].

Several authors have been reported the presence of heavy metals (e.g., lead [2,3,4], mercury [3,4], arsenic [5], thallium [5], aluminum [6,7], chromium [8], nickel [9] and cadmium [9,10]) above of the limits allowed in the pharmaceutical products.

The heavy metals test recommended by United States Pharmacopoeia, and similar tests provided in the European or British Pharmacopoeia consist of the precipitation of metal sulfides from aqueous solution and the visual comparison of the color of the precipitate, which is suitable for the determination of ions giving black or dark color sulfides. Substances that typically will respond to this test are lead, mercury, bismuth, arsenic, antimony, tin, cadmium, silver, copper and molybdenum but no information is given about the concentrations of individual elements. Therefore this method supply few qualitative and quantitative information, and various toxic metals can not be determined (such as aluminum, selenium, tellurium, chromium, nickel and others). From this point of view recent publications [11,12,13] have been shown the need to change methodology to instrumentals methods of analysis.

For the analysis of drugs based on antimony compounds like the used for leishmaniasis treatment the recommended procedure for heavy metals analysis is prone to several kinds of interference. In this case the presence of a high level of antimony makes the correct evaluation practically impossible.

Based on the above considerations the aim of this work was to perform the determination of As, Bi and Pb (at ppm level or less) in meglumine antimonate (pharmaceutical products and raw material) using instrumental method of analysis: flame atomic absorption spectrometry (F AAS) and inductively coupled plasma mass spectrometry (ICP MS). The choice of As, Bi and Pb was made due to recent newspapers reports on the possibility of contamination of commercial products by these elements.

Experimental

Measures were carried out by F AAS and they were performed with an atomic absorption spectrometer (Perkin-Elmer model 3030). The background correction was made with a deuterium lamp. The wavelength selected for the elements was: λ_{Pb}= 283.3 nm, λ_{As}= 190.7 nm and λ_{Bi}= 223.1. All measurements were made in integrated absorbance mode. The ICP MS measurements were performed in a Perkin-Elmer-Sciex (model ELAN 6000) with a pneumatic nebulization.

Results and discussion

In this work, raw material and pharmaceutical products available for use in Brazil were evaluated using F AAS and ICP MS as method of analysis. Results are shown in Table 1 (ICP MS determination). No significant differences were observed for the results from F AAS method (Table 2) in comparison with those from ICP MS. Thus, both instrumental can be used to determination of As, Bi and Pb in meglumine antimonate.

Table 1
Heavy metals determination by ICP MS in meglumine antimonate injectable dosage form (samples “A” and “B”) and raw material (sample “C”)*

	A (mg l^{-1})	**B** (mg l^{-1})	**C** (µg g^{-1})
As	3.45 ± 0.31	46.31 ± 1.64	103.5 ± 5.8
Bi	0.37 ± 0.08	4.90 ± 0.37	60.87 ± 3.39
Pb	< 0.10	52.31 ± 1.07	209.4 ± 5.9

* results represent the mean and the standard deviation of the measures (n=5).

Table 2
Heavy metals determination by FAAS in meglumine antimonate injectable dosage form (samples “A” and “B”) and raw material (sample “C”)*

	A (mg l^{-1})	**B** (mg l^{-1})	**C** (μg g^{-1})
As	3.32 ± 0.25	46.15 ± 1.20	103.8 ± 3.2
Bi	0.38 ± 0.07	4.83 ± 0.26	60.67 ± 2.21
Pb	< 0.20	52.39 ± 0.85	207.5 ± 4.6

* results represent the mean and the standard deviation of the measures (n=5).

Conclusion

Both the instrumental methods proposed in this work (ICP MS and F AAS) can be used to determination of arsenic, bismuth and lead in meglumine antimonate. The analytical performance of these methods is more suitable to heavy metals control due the possibility of individual quantitative information for each determined element. Therefore these two methods can be proposed for analysis of pharmaceutical products and raw materials based on antimony compounds. Results for some selected commercial products show high levels of arsenic and lead in one of injectable sample and in the raw material.

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AM-09 STUDY OF POLYMORPHISM IN ANTI-INFLAMMATORY DRUGS

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Introduction

Thermal analytical techniques have been used to characterize the behavior of the sample as function of temperature. These methods, including differential scanning calorimetry (DSC), have extensively been applied to identify polymorphism in drugs [1].

A polymorph is a solid crystalline phase of a given compound, resulting from at least two different molecular arrangements in the solid state [1,3]. Thus polymorphs can have different melting points, density, solubility, dissolution, bioavailable and physical and chemical stability. Polymorphs are divided at two groups: enantiotropic when the phase transition is reversible and monotropic when the phase transition is irreversible [2].

In the preset work DSC was used to determine the polymorphism in drug anti-inflamatory (sodium diclofenac, acetaminophen and prednisone).

Experimental Methods

- **Materials**

The sodium diclofenac drug A was acquired in the LTF/UFPB source from Chine. The drug B was donated by FURP (Fundação para o remédio popular). The paracetamol drug was acquired in the LTF/UFPB source from Chine. The prednisone drug A was acquired in the LTF/UFPB source from Chine. The drug B was donated source from German. And the drug C was donated source from Virgin Island

- **Techniques**

The DSC curves were obtained with a Shimadzu model DSC-50 calorimeter, in the temperature range 25 – 500ºC, under an atmosphere of nitrogen at a flow rate of 50 mL min^{-1}. The samples were analysed at a heating rate of 1, 2, 4, 8, 16, 24 and 40ºC min^{-1}. up to 400ºC. The prednisone drugs A and B also were analysed at a heating rate of 5, 10, 20ºC min^{-1} up to 500ºC.

Results and Discussion

The DSC curve of sodium diclofenac, drug A, at a heating rate at 10ºC min^{-1} contained an endothermic peak corresponding to melting point at 291ºC, which is higher than the range reported in the literature: 280 – 285ºC [4]. The drug was submitted to heating at 5ºC min^{-1} what has found that the melting point of sodium diclofenac at 285ºC. The drug also was submitted two heating run (Figure 1). The present figure shows that the endothermic peak is irreversible, a case of monotropy.

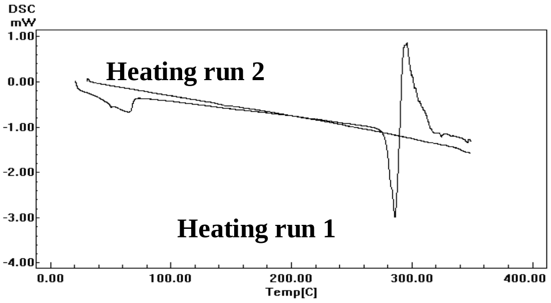

Figure 1 – The two heating run of diclofenac sodium, drug A.

The sodium diclofenac drug (A and B) were therefore submitted to heating rates: 1, 2, 4, 8, 16, 24 and 40 ºC min^{-1}. However, sodium diclofenac drugs displayed significant differences in melting point at the different heating rates, which influenced the curve shape (Table 1).

Table 1 – Parameters of polymorphism study of diclofenac sodium drug A and B.

Drugs	Rate Heating. (ºC/min.)	$T_{m/ºC}$*	$\Delta H_f^o/_{Jg-1}$**
A	1	268.44	15.11
	2	275.05	36.08
	4	281.45	60.16
	8	287.81	64.46
	16	292.61	79.94
	24	298.69	67.06
	40	302.91	75.74
B	1	271.26	32.90
	2	275.45	41.55
	4	281.68	67.18
	8	288.77	77.73
	16	294.48	74.29
	24	302.75	69.59
	40	302.56	92.14

* Temperature of melting point, ** Heat fusion

The DSC curve of acetaminophen drug at a heating rate at 10ºC min^{-1} contained an endothermic peak corresponding to melting point at 170ºC which is reported in the literature: 169.0 – 170.5ºC [4]. The drug was submitted a two heating run which also showed an endothermic peak at 157.72 ºC, a case of enantiotropy (Figure 2). It was made a study to heating at different heating rates which displayed insignificant differences in melting point (Table 2).

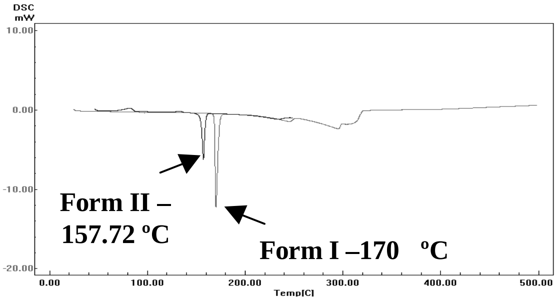

Figure 2 – The two heating run of paracetamol drug.

Table 2 – Parameters of polymorphism study of paracetamol drug.

Heating rate (ºC/min)	**Peak (ºC)**	**Heat (kg/kj)**
1	170.21	158.36
2	169.68	167.68
4	170.50	139.85
8	170.84	168.30
16	172.17	165.40
24	172.40	163.38
40	172.94	172.62

The DSC curve of prednisone drugs, A and B, at a heating rate at 10 ºC min^{-1} contained an endothermic peak corresponding to melting point at 239 and 240 ºC, respective, which is higher than the range reported in the literature: 233 – 235 ºC [4]. The drugs were therefore submitted to heating at different rates: 2, 5, 10 and 20 ºC min^{-1} (Table 3). As concern suitable choice of heating rates, it was found that the melting point of prednisone could be determined correctly only at a heating rate of about 5 ºC min^{-1}. The prednisone drugs also were submitted two heating run (Figure 3) and also heating rates: 1, 2, 4, 8, 16, 24 and 40 ºC min^{-1} (Table 4). The figure 3 shows that the endothermic peak is irreversible, a case of monotropy. However, prednisone displayed significant differences in melting point at the different heating rates, which influenced the curve shape.

Table 3 – Prednisone drug data at different heating rate.

Rate Heating. (ºC/min.)	$T_{m/ºC}$*	$\Delta H_f^o/_{Jg\text{-}1}$**
2	228.45	105.55
5	233.09	83.11
10	239.30	100.07
20	245.47	98.91

* Temperature of melting point, ** Heat fusion

Table 4 – Parameters of polymorphism study of prednisone drugs.

Drugs	Rate Heating. (ºC/min.)	$T_{m/ºC}$*	$\Delta H_f^o/_{Jg\text{-}1}$**
A	1	220.45	107.79
	2	223.67	104.80
	4	236.79	114.10
	8	236.98	95.59
	16	243.67	102.13
	24	246.18	98.41
	40	247.38	68.48
B	1	225.39	100.06
	2	227.68	106.03
	4	235.60	75.05
	8	240.15	99.03
	16	242.00	84.70
	24	245.36	83.31
	40	250.76	85.50
C	1	226.24	99.24
	2	230.68	98.97
	4	232.18	105.65
	8	238.07	99.42
	16	244.92	97.53
	24	246.72	97.61
	40	247.48	82.56

* Temperature of melting point, ** Heat fusion

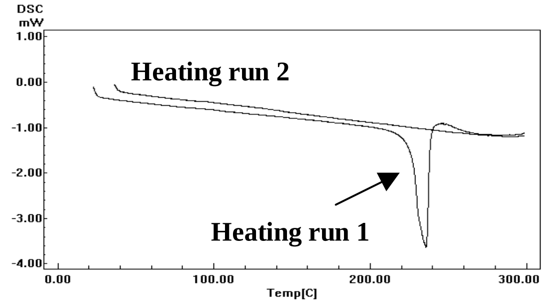

Figure 3 – The two heating run of prednisone drug .

Conclusion

Two studied raw materials, sodium diclofenac and prednisone, presented irreversible polymorphs whereas paracetamol did not present any polymorph.

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AM-10 APPLICATION OF THERMAL ANALYSIS IN THE CHARACTERIZATION OF CEPHALEXIN

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Introduction

The cephalexin is an antibiotic of the cephalosporanic group used in the therapeutic, mainly in the cases of infection by *S. aureus* [1]. Thermal analysis are a group of related techniques that study the analytical and kinetic parameters of a substance in function of temperature [2]. The pre-formulation study may be described as a phase of the development process where are characterized the physical, chemical and mechanical properties of a drug, in order to develop stable, safe and effective dosage forms [3].

The present work proposes to evaluate a chemistry stability of cephalexin in comparison with three formulations, utilizing the methods of differential scanning calorimetry coupled to photovisual system and thermogravimetry.

Materials and methods

Materials

Cephalexin, avicel 101, magnesium stearate, explocel, starch, PVP, tablet A (LTF/UFPB), tablet B (reference sample) and tablet C (generic sample).

Methods

- **Preparation of binary mixtures and tablets**

The binary mixtures were sifted in 100mesh, and conditioned in brown container. The tablet A shows cephalexin-78.74%, avicel 101 – 8.66%, starch – 5.35%, PVP – 4.1%, explocel – 2.21%, magnesiun stearate – 0.95%. The tablets A, B and C were pulverized and sifted in 100mesh and conditioned in brown flask.

- **Differential Scanning Calorimetry coupled to Photovisual System**

The cephalexin, tablet A, B, C and binary mixtures were analyzed in a Shimadzu Calorimetrer, model DSC-50, coupled to photovisual system, under nitrogen flow 50mL/min, heating rate 10ºC/min, until 500ºC, using a mass about 2 mg in aluminum cell.

- **Thermogravimetric Analysis**

The non-isothermal curves were obtained with a Shimadzu termobalance, model TGA-50, under an air atmosphere with a constant flow of 20mL/min, under nitrogen flow 50ml/min, at a heating rate of 10 ºC /min, until the temperature of 900ºC, using a mass of around 8 mg in an alumina cell.

The isothermal thermogravimetric curves of cephalexin, tablets A, B and C were obtained in temperatures of 190, 180, 170, 160 and 150 ºC. The isothermal TG data were analyzed by a software TASYS Shimadzu.

The rate constants were calculated from isothermal TG data using Arrhenius classical equation.

Results and Discussion

The DSC curves of cephalexin drug, tablets A, B, C and binary mixtures: cephalexin-starch, cephalexin-PVP, cephalexin-magnesium stearate, cephalexin-avicel presented four phase transitions. Only the binary mixture cephalexin-explocel presented five phase transitions. (Figure 1)

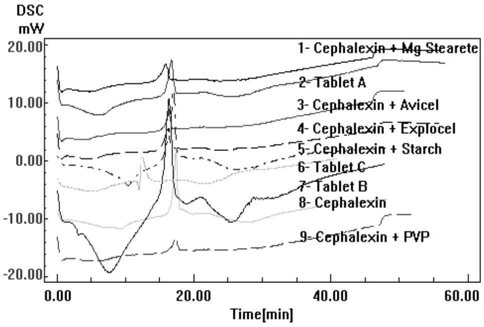

Figure 1 – DSC curves of cephalexin, binary mixtures, tablets A, B and C.

The DSC curves were made in different heating rate to determine the existence or not of pseudo-polymorph in the cephalexin. The Figure 2 showed small change in the peak temperatures evidencing only polymorph in the cephalexin.

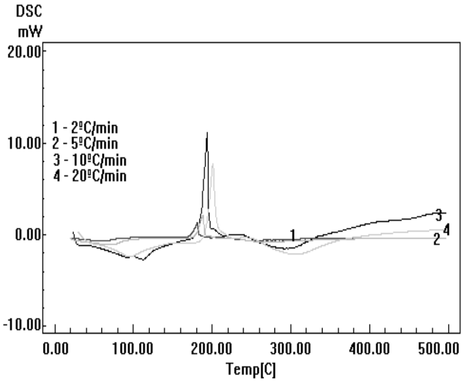

Figure 2 – DSC Curves of cephalexin in the heating rate of 2, 5, 10 and 20 ºC/min.

The DSC data of the cephalexin, binary mixtures, tablet A and tablet B revealed the cephalexin does not present characteristic melting peak Figures 1 and 2. This fact was confirmed by DSC-photovisual (Figure 3). The cephalexin is yellow at 180ºC, then it acquires plastic nature and before the melting, the cephalexin decomposes at 193ºC, followed by gas formation. The tablet A is yellow at 182ºC, it decomposes at 189ºC with gas formation too. But tablet B is different, it starts decomposition at 192ºC and it finishes at 200ºC. The thermal behavior of tablet C is similar the cephalexin and tablet A, but its decomposition was anticipated to 162ºC, with decomposition whole in 235ºC.

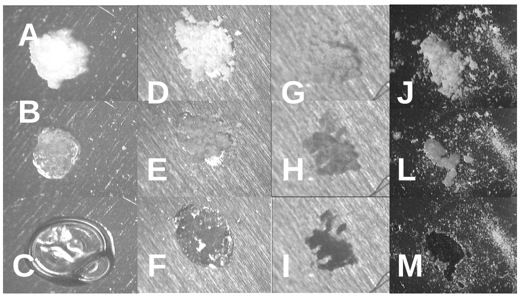

Figure 3 – DSC photovisual of cephalexin (A – room, B – 193ºC and C – 211ºC), tablet A(D – room, E – 188ºC and F – 200ºC), tablet B (G – room, H-192ºC and I-200ºC) and tablet C (J- room, L- 162ºC and M-235ºC).

The TG curves (Figure 4) cephalexin, binary mixtures, tablet A, B and C presented five decomposition stages, where stage first corresponds the water loss and stages other correspond the decomposition.

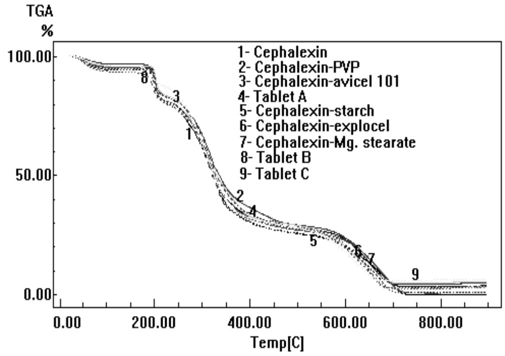

Figure 4 – TG curves of cephalexin, binary mixtures, tablets A, B and C.

The kinetic data of the decomposition reaction of cephalexin obeyed a first order kinetic. The rate constants had stability similar to the tablets (Table 1). However o tablet B presented more stability when compared to cephalexin drug, tablets A and C.

Table 1 – Rate constants of the cephalexin, tablets A, B and C.

Temp .(ºC)	Rate Constants (k^{-1})			
	CEPHALEXIN	TABLET A	TABLET B	TABLET C
190	$8.27x10^{-05}$	$4.78x10^{-05}$	$3.93x10^{-05}$	$6.24x10^{-05}$
180	$3.08x10^{-05}$	$2.82x10^{-05}$	$2.37x10^{-05}$	$5.78x10^{-05}$
170	$1.56x10^{-05}$	$1.64x10^{-05}$	$7.78x10^{-06}$	$1.21x10^{-05}$
160	$2.66x10^{-06}$	$1.24x10^{-05}$	$3.44x10^{-06}$	$4.25x10^{-06}$
150	$2.37x10^{-06}$	$1.12x10^{-05}$	$2.85x10^{-06}$	$1.19x10^{-06}$

Conclusion

The cephalexin tablets showed differences among them in the thermal behavior, evidencing different pharmaceutical formulations.

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AM-11 ANALYSIS OF CALCIUM CONTENTS IN TABLETS AND DISSOLUTION MEDIA BY MEANS OF ICP-AES

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Introduction

Since the 70's, the study of minerals has improved due to the development of more sensible and accurate analytical methodologies, which allowed the determination of elements found in food and biological fluids as well as to find out the mechanisms that are used by minerals in order to hold their function in the organism. The researchers have been concerned about an accurate definition of the organism's needs and the establishment of alimentary ingestion's measures though knowledge of biodisponibility. This has caused serious discussion into the scientific community, in order to establish the Recommended Dietary Intake (RDI)[1-3] , since it is intended to found the ideal level to prevent the chronic diseases, not only the minimum necessary.

The bioactive products are responsible for important biological/pharmacological activities in human or animal organisms that receive them. The quality of these bioactive products is essential in order to certify the achievement of the desired effect. The bioactive products wealthy in minerals have been marketed in the national and world business without registered data that assure their quality [1].

At the least years, the analytical instrumentation for metal determinations have been suffered considerable advances. The Inductive Coupled Plasma – Atomic Emission Spectrometry technique (ICP-AES) have been amply used by the advantages that offer, as: simultaneous multielementar analysis, sensibility, accurate and rapidity, as well as vast linear dynamic interval.[2-4]

This work intends to develop a method to determine the calcium contents seeking to verify the release of this mineral from tablet.

Experimental methods

- **Apparatus**

  a) Microwave Digestor (Provecto System – DGT 100)
  b) ICP-AES System (Varian – Libert series II)
  c) Dissolutor (Vankel – VK7010)

- **Materials**

  a) Calcium standard, Merck
  b) Nitric acid 65%, Merck
  c) Hydrogen peroxide 30%, Merck
  d) Deionized water

- **Standard Solution**

From calcium tritisol stock solution 1,000 ppm, were prepared standard solutions containing 0.25, 0.50, 1.0, 1.5 and 2.0 ppm.

- **Sample Preparation**

Microwave Digestion

Ten tablets of calcium, acquired in the market of João Pessoa – Paraiba – Brazil, were weighed and finely powdered. Eighteen aliquots containing 500mg of powder, equivalent to about 174 mg Ca, were weighed and transferred to digestion vessels. 3.0 mL HNO_3 were added to each sample and 0.5 H_2O_2 to each digestion vessel. Conditions of microwave operation: 1) first step: 400W for 3 min.; 2) second step: 790 W for 5 min.; 3) third step: 000 W for 3 min. Upon completion of microwave heating, allow samples to cool for 20 min. and vent pressurized vessels before removing vessel caps. The stock solution was prepared by vessels contents what were transferred to volumetric flask of 50 mL and its volume completed with deionized water. The final solution was prepared with 25 µL of stock solution in 50 mL of deionized water.

Dissolution

Were weighed 18 tablets, individually, and transferred to vessels of dissolution test. The assays were conducted with different two dissolution medias and conditions. The first was in water pH 2.0 with HCl and the second was in water pH 3.5 with HCl and KCl. The dissolution tests were effected with apparatus 1 at 100 rpm (medium pH 2.0) and apparatus 2 at 50 rpm (medium pH 3.5), according USP 23 [5]. The samples were collected after 30 and 60 minutes, filtered in 0.45 µm. The final solution was prepared by dilution of 100 µL of dissolution media in 20.0 mL of deionized water.

- ICP-AES

The plasma was initialized and the system was allowed to equilibrate thermal over 10 min. Calibration and standardization were performed per manufacturer's specifications with standards 0.25, 0.5, 1.0, 1.5, 2.0 ppm of calcium (n=10). The emission intensity of calcium was measured at 393.366 nm. Twenty four determinations were effected to each sample.

Results and Discussion

- **Calcium Contents**

The tablets powder submitted to digestion presented average value of 1.8231 ± 0.0738ppm of calcium in the solution. The calcium contents in tablets was calculated and showed an average value of 630.1 ± 25.5 mg or 105.02% of labeled value.

- **Dissolution studies**

The dissolution data of calcium contents in tablets are given in table 1. In pH 2.0 the samples presented a more release of calcium in relation to pH 3,5. All samples dissolved slowly, where best release of calcium were exhibited at 60 min. in pH 2.0. The data analyses showed that the calcium released in best situation was 18.4% of labelled value. Such fact reveals the necessity of the dissolution studies of these products.

Table. 1
Dissolution studies

Time (min.)	Medium	Calcium Tablet (mg)	Calcium release (%)
30	pH 2.0	98.2	16.4
60	pH 2.0	110.1	18.4
30	pH 3.5	27.4	4.6
60	pH 3.5	23.9	4.0

Conclusion

The calcium contents in tablets showed 105.02% of labeled value. However, only 18.4% of calcium was released in the dissolution studies.

Acknowledgments

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AM-12 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF CEFETAMET PIVOXIL HYDROCHLORIDE IN TABLETS

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Introduction

During the last few years, one of the major advances in the field of antimicrobial therapy has been in the area of oral derivatives of the aminocephalosporanic ring, the cephalosporins [1]. Cephalosporins are among the safest and the most effective broad spectrum bactericidal antimicrobial agents available to the clinician, and have therefore become the most widely prescribed of all antibiotics [2]. Cefetamet pivoxil hydrochloride (Fig.1) is an orally absorbed prodrug ester of the microbiologically active cephalosporin, cefetamet [3]. Cefetamet is classified as a third generation cephalosporin wich has excellent in vitro activity against a wide range of Gram-negative and Gram-positive community acquired pathogens, and posses a very high intrinsic stability to the hydrolitic action of the most common β-lactamases and penicilinases [3,4]. Cefetame pivoxil hydrochloride is available in tablet and syrup dosage forms. Chemically it is [6R- [(6α,7β(Z)]] -7-[[(2-Amino-4-thiazol) (methoxyimino) acetyl]amino]-3methyl–8-oxo-5-thia-1-azabyciclo [4.2.0] oct-2-ene-2-carboxylic acid [5] and is not yet official in any pharmacopeia. Two methods were described for the determination of cefetamet in biological fluids and plasma by high-performance liquid chromatography (HPLC) [6,7]. HPLC methods are very useful in the determination of drugs. We believe that the availability of this new method, with increased sensitivity, selectivity, will be very useful for the determination of cefetamet pivoxil hydrochloride in pharmaceutical preparations. The aim of this study was to develop a simple, rapid and reproducible reversed-phase HPLC method for the quantification of cefetamet pivoxil hydrochloride in raw material and tablets.

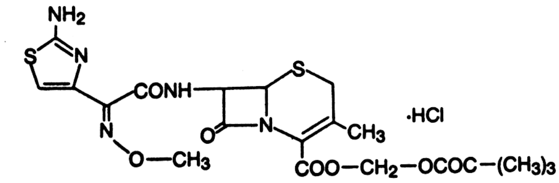

Fig. 1 – Chemical structure of cefetamet pivoxil hydrochloride

Experimental

Chemical and reagents

The cefetamet pivoxil hydrochloride reference substance (assigned purity 99.57%) was generous supplied by Roche Laboratories, while tablets were obtained commercially. Tablets were claimed to contain 500mg of cefetamet pivoxil hydrochloride. All other chemicals used were of analytical grade. Water was glass-distilled.

Apparatus and chromatographic conditions

The HPLC system consisted of a Shimadzu SCL-10 A-VP, equipped with a model LC-10 AD-VP pumps; and SPD 10A-VP, variable-wavelength UV-Vis detector (set at 265nm), an SCL-10A system controller; a Class VP software package integrator and Rheodyne injection valve with a 20μL loop (Shimadzu, Kyoto, Japan). A shim-pack CLC-ODS (M) column (150.0 x 4.6mm i.d., 5μm particle size). The mobile phase was a mixture of acetonitrile:water (60:40 v/v) pH 4,4 adjusted with acetic acid solution 10%, isocratic as the mobile phase, at flow rate of 1.0ml min^{-1}. The sensitivity was 1.0 AUFS. The HPLC system was operated at ambient temperature (20 ± 1°C).

Procedure

Cefetamet pivoxil hydrochloride reference standard

The cefetamet pivoxil hydrochloride standard (25mg) was weighted accurately and transferred into a 25ml volumetric flask and diluted with mobile phase. From this solution, aliquot (10ml) was transferred volumetrically into 100ml volumetric flask and mobile phase added to make up to volume to yield stock solution (100μg ml^{-1}). Appropriate amounts of the stock solution were diluted with mobile phase yielding concentrations of 20, 24, 28, 32, 36 and 40μg ml^{-1}. Triplicate injections of each were made.

Assay of tablets

The tablets were weighted and pulverized. An amount of power equivalent to 500mg of cefetamet pivoxil hydrochloride was transferred to 100ml volumetric flask with 50ml methanol and shaken for 30min, followed by adding methanol to make up the volume. This sample solution had a concentration of 5mg ml^{-1}. After filtration, the dilutions were made with mobile phase to give a final concentration of 24μg ml^{-1}. All determinations were conducted in triplicate.

Calculations

Having established the quantitative relationships between the parameters studied, and knowing the predictive performance of their association model, a linear simple regression by the least squares method was applied.

Recovery of cefetamet pivoxil hydrochloride tablets

The recoveries were determined by adding know amounts of the cefetamet pivoxil hydrochloride reference substance (8, 12 and 16μg ml^{-1}) to the samples at the beginning of the process. A recovery exercise was then performed. The recovery test and precision data for commercial tablets are show in Table 1.

Results and discussion

The development of methods in HPLC for the determination of drugs has received considerable attention in recent years because of their importance in quality control in pharmaceutical analysis. Validation of the methods was carried out using parameters, such as calibration curve, linearity, recovery, precision and accuracy [7]. The goal of this study was to develop an HPLC assay for the analysis of cefetamet pivoxil hydrochloride in raw material and tablets. In this study, the chromatographic conditions were influenced by the physic-chemical properties of cefetamet pivoxil hydrochloride, such as solubility, polarity and UV absorption. The retention time (t_R) of cefetamet pivoxil hydrochloride was about 2.95min. The HPLC chromatogram shows t_R (Figure 2).

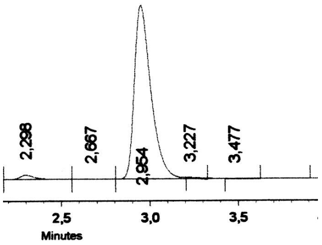

Figure 2-Chromatogram of cefetamet pivoxil hydrochloride at 24μg ml

The calibration curves for cefetamet pivoxil hydrochloride were constructed by plotting concentration versus peak area and showed good linearity in the 20-40μg.ml^{-1} range. The representative linear equation was $y=-2561 +27807x$ (n=6, r=0.9999). The method was validated by evaluation of intra and inter-day precision. Relative standard deviation of assay method precision was found to be between 0.38 to 0.91%. Recovery studies gave results between 100.63 to 101.75%. No interference from the sample solvent, impurities and dosage form excipients could be observed at the detection wavelength (265nm).

Table 1
Experimental values obtained in the recovery test for cefetamet pivoxill hydrochloride tablets by HPLC.

	Amount of standard (μg ml^{-1})			
	Added	Recovered	Recovery[a] (%)	RSD (%)
R1	8.0	8.05	100.63	0.28
R2	12.0	12.12	101.00	0.13
R3	16.0	16.28	101.75	0.15

[a] Mean of three replicate analysis.

Conclusions

The present study proposes a simple, rapid and precise HPLC method for determination of cefetamet pivoxil hydrochloride in pharmaceutical dosage forms and may be used as stability-indicating method.

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AM-13 DETERMINATION OF Sb (III) IN ANTIMONIAL DRUGS

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Introduction

Leishmaniasis is a chronic inflammatory disease caused by parasitic protozoa of the genus *Leishmania sp.* The first preparations were based on trivalent antimonial compounds, Sb (III). Because its toxic effects, a second generation of antimonial drugs were developed based on pentavalent antimony, Sb(V) [1]. Today, pentavalent antimonials in the form of meglumine antimonate and sodium stibogluconate are the drugs of choice for treatment of leishmaniasis [2].

Nowadays, the only drug available for use in Brazil is meglumine antimonate. The injectable form is a solution containing the equivalent of 85 mg of Sb(V) in a mililiter [2]. However, there are no official tests in Pharmacopoeias describing the determination of this specie in meglumine antimonate. In addition, the possibility of Sb (III) to be present as a contaminant in this drug makes important its determination in view of the higher toxic effects of Sb (III) in comparison to Sb (V).[1,3] The determination of Sb (III) isn't an easy task and several kinds of interference and other related problems are referred in the literature.[4] Hydride generation atomic absorption spectrometry (HGAAS) is a very powerful technique to determination of elements and can be used to speciation studies.

The aim of this study was to develop a selective procedure for Sb (III) determination by HGAAS in meglumine antimonate formulations. The main goal for this procedure is the possibility of determination of Sb (III) in the presence of high concentration of Sb (V). In addition, the determination of total Sb was performed by two procedures: flame atomic absorption spectrometry (FAAS) and iodometric titration [1]. The Sb(V) concentration was obtained by difference among the value found for total Sb and Sb (III).

Experimental

- **Instrumentation**

All atomic absorption determinations were made using an atomic absorption spectrometer Perkin-Elmer (Model 3030), equipped with a hydride system (Sb III determination) or air/acetylene flame system (total Sb determination). The background correction was made using a deuterium lamp. The wavelength selected was 217.6 nm (slit = 0.7 nm). All determinations were made in integrated absorbance mode.

- **Reagents**

Analytical solutions for Sb (III) and total Sb were prepared by serial dilutions (in water) from Sb_2O_3 in 6 mol l^{-1} HCl and $SbCl_3$ in 6 mol l^{-1} HCl solutions, respectively. All the reagents were at least of analytical grade and water was distilled and doubly deionized.

- **Sample preparation**

a) Sb (III) determination: meglumine antimonate drugs were diluted with water 10,000 times.
b) total Sb determination: meglumine antimonate were diluted with water about 2,500 times.

Results and discussion

The selective determination of Sb (III) in the samples of meglumine antimonate was possible by optimization of reacional conditions: the kind and concentration of reacional medium, pH, sodium tetrahydroborate ($NaBH_4$) concentration, injection time, and purge time.

The chosen reacional conditions were: 4% (m/v) citric acid, 1% (m/v) $NaBH_4$, purge time of 10 s, injection time of 10 s. Citric acid allowed the Sb (III) determination in a presence of an excess of 2,000-fold of Sb (V). Results for Sb (III), total Sb, and the comparison of procedures for total Sb determination are show in Table 1 and Table 2, respectively.

Table 1. Results (mg.ml^{-1}) obtained for Sb (III) and total Sb determination (AAS) in samples of meglumine antimonate (n=5)

	Sample A	Sample B
Total Sb	88.90 ± 0.81	83.91 ± 0.92
Sb (III)	1.71 ± 0.11	1.67 ± 0.13
Sb (V)	87.19 ± 0.81	82.24 ± 0.93

Table 2. Determination of total Sb by FAAS and titrimetric procedure, mg.ml^{-1} (n=5)

Procedure	Sample A	Sample B
F AAS	88.90 ± 0.81	83.91 ± 0.92
Titrimetry	82.85 ± 2.93	81.45 ± 2.84

For the determination of total Sb, the difference between the results by the two procedures can be attributed to precipitation effects occurring during the determination of Sb by the titrimetric procedure. This fact results in lower values by this procedure. To ascertain if the FAAS procedure is prone to matrix effects and to verify the accuracy a recovery test was made by the spike of Sb reference solutions to the samples. Additionally, the recovery of Sb (III) was also made for the same samples. Recoveries varied from 99 to 101% and from 98 to 100.5% for total Sb and Sb (III), respectively.

The proposed procedure by HGAAS for Sb (III) determination was applied to analysis of commercial samples of meglumine antimonate. The concentration of total Sb varied from 82.6 to 116.8 mg.ml^{-1} and from 1.56 to 3.66 mg.ml^{-1}.

Conclusion

The determination of Sb (III) by the HGAAS procedure was simple, rapid and requires minimal sample preparation. The proposed method shown good sensibility and high selectivity allowing the determination of Sb (III) in the presence of as high excess of Sb (V) as 2,000 fold. Determination of total Sb by FAAS shown lower standard deviation than the titrimetric procedure. Thus, the proposed procedure may be used for Sb (III) and total Sb determination in antimonial drugs.

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AM-14 VALIDATION OF THE ANALYTICAL METHODOLOGY OF ANTI-RETRO VIRAL MEDICATION THROUGH HPLC

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Introduction

Zidovudin, a synthetic composite, has systematic anti-viral and virulent action. It is also known as AZIDOTIMIDIN (AZT), an anti-retro viral drug, active against the virus HIV. It is absorbed rapidly and nearly totally by the gastro-intestinal track, after oral administration. Its systematic biovailability is approximately 65%. It is recommended as a primary agent in the treatment of adult patients suffering from sickness caused by human immune-deficiency virus.

Chromatography is a generic term applied to the process of physical-chemical separation, which is based on phenomenon of absorption and division. Due to its great importance as a drug selected in the treatment of AIDS, interest in its analysis has increased, especially within the Ministry of Health.

Validation, in analysis, views the analytical trustworthiness of the laboratory and of the method selected. Validation of the methodology using high-performance liquid chromatographic (HPLC) in the analysis of AZT is based on the following information. It is a selective method; it is precise and exact. It is used with innumerable drugs and used in the main, official codes and by the main pharmaceutical industry.

The aim of our work was to validate the methodology for the analysis of zidovudin and introduce it in the Quality Control Laboratory of the Project REBLAS and Brazilian Pharmacopoeia.

Experimental Method

- **Material and Equipment**

Chemical substance for primary reference: zidovudin – Osvaldo Cruz Foundation
Chemical substance for secondary reference: zidovudin – FURP
Reactants – HPLC degree and flasks
Chromatography: Shimadzu SCL 10 AVP with a C18 reversed phase column
Potentiometer: Water purifier, analytical balance.

- **Methodology**

The method of analysis is based on the chromatographic detection of zidovudina by chromatography of division by reverse phase, using isocratic conditions.

- **Preparing the standards**

Nearly 10mg were weighed analytically of the standard AZT and were transferred to the volumetric balloon (flask) of 100ml. It dissolved and the volume was completed using HPLC degree methanol. It was homogenized for 10 minutes. After this, dilutions were made during a mobile phase in concentrations using a working ratio (0.01mg/mL to 0.05mg/mL).

- **Chromatographic conditions**

Column: C18 Merck Pre-Packed Column RD 125-4
Wave length: 267nm
Flow: 1.0 mL/min
Running time: 10 minutes
Retention time: 6 minutes
Mobile phase: phosphate buffer solution and HPLC degree methanol (780:220) with a pH of 6.7± 0.2.

50 µL of the substance to be analyzed was injected into the chromatographic liquid. The chromatogram was registered and the operation was repeated, thus guaranteeing the reproducibility of the analysis.

Results and discussion

Table 1. Average results obtained from primary reference AZT substance (SRP) and the secondary one (SRS) and the phosphate correlation coefficient linear (CCL) of both of the curves.

Conc. mg/mL	SRP	CCL	SRS	CCL
0.01	493700	0.9999	502933	0.9999
0.02	1000721		1006110	
0.03	1489966		1502001	
0.04	2003383		2010851	
0.05	2494930		2522023	

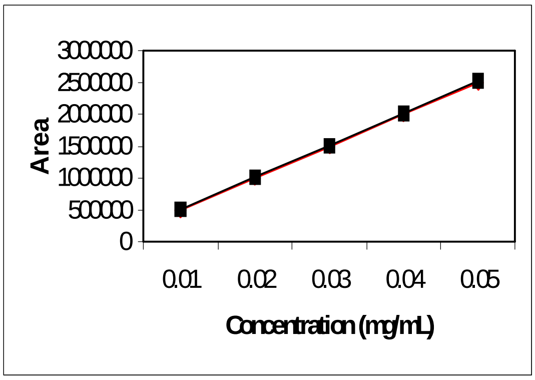

Figure 1. Comparison of the linearity of the curves of the primary reference substances (SRP) and the secondary reference substances (SRS), area vs. concentration.

In table 1 the results of the analyses show areas with very close values, thus being able to compare the precision of the method for both the standards. The repeatability in the data of area and concentration is observed in the analyses between days, resulting in low coefficient of variation. A variation co-efficient of 0.59% for SRP and 0.64% for SRS.

It was possible to observe that the averages of the samples do not vary significantly, with a level of significance being 95%. This proves the reproducibility of the analysis over consecutive days.

Figure 1 shows the linearity curves of both reference standards. By making this comparison it was possible to verify the linearity of the two, besides showing that there was no significant difference in the angular coefficient of both.

The coefficient of the linear correlation showed that the variations of the area are quite explicit by the regression and that the results indicate that the system presented a significant correlation.

Conclusions

The analytic method for zidovudin through chromatographic liquid of high efficiency presents results that are precise, repeatable and reproducible, having no significant differences were observed by comparing the results between the data obtained.

The results obtained from the coefficient of variation, showing values within the allowed limits, demonstrates the reproducibility of the method. Thus the method can be used routinely in the laboratory quality control assay for the quantitative determination of zidovudin .

The validation of the analytical methods takes the investigations of quality control to the aspect of security. This, besides substantially contributing to its inclusion in Brazilian Pharmacopoeia as an alternative to analytic assay.

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AM-15 VALIDATION OF THE ANALYTICAL METHODOLOGY FOR STABILITY EVALUATION OF LAPACHOL IN SOLUTION

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Introduction

The lapachol is a naphtoquinone found in several species of the *Bignoniaceae* family, especially those of the gender *Tabebuia*. Chemically it is 2-hydroxy-3-(3-methyl-2-butenil)-1,4-naphtalenedione (Figure 1), possessing antimicrobial activity against bacteria and fungi, antiviral, anti-inflammatory and mainly anticancer activity [5].

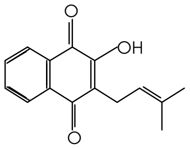

Figure 1 – Chemical structure of lapachol.

Due to its pharmacological properties it has been thoroughly studied to be used as a therapeutic agent. As in initial step to a pharmaceutical development study as a drug it is necessary to use a validated analytical methodology and to verify the factors that influence its degradation. The aim of this work consist of the development and validation of analytical methodology for the lapachol and its preparations, and its application in the study of the factors that contribute to the degradation of its solutions and the quantification of the intensity its action.

Experimental Methods

Lapachol Sigma, Batch: 33:34575, β-Lapachone prepared by the Institute of Antibiotics of UFPE, methanol UV/HPLC grade, distilled water processed by Milli-Q for HPLC use. All other reagents and solvents were of analytical grade.

System of high performance liquid chromatography HP-series 1100 (Hewlett Packard), composed by degaser, quaternary pump, manual injector with loop of 20μL, chromatographic column Lichrosorb RP-C18 250x4mm (Merck), UV detector and integrator.

For analytical methodology validation it was initially looked for the best wavelength for the lapachol detection, among the three cited absorbance maxima at: 251, 278 and 331nm [6]. These values were confirmed according the validation criteria, according to the norm ICH topic Q-2b, 1996 [3].

The stability evaluation of the lapachol solutions was determined in relation to pH (1.9; 5.0 and 7.4), light, and temperature (25.2; 37.0 and 50.0ºC). We also observed the effect of the polyvinyl alcohol (PVA) presence on the lapachol stability in solution.

Results and Discussion

The chromatographic conditions used were as follow: mobile phase: methanol - 5% acetic acid aqueous solution (80:20); flow rate: 1mL/min; column Hibar Lichrosorb RP-18 250x4mm; injection volume of 20 μL.

From the wavelengths of maxima absorbance for the lapachol, the 331nm gave the smallest value of the extinction coefficient, and the 251nm the biggest one, however with high variation among the readings taken for the same concentration. The variation coefficient of the repeatability at 278nm, with n=6 was only 0.08%, and of the reproducibility within days and within analysts didn't present significant differences at the level of 95% of confidence, with p=0.08 and 0.13, respectively.

The linearity was verified from 0.1 to 20μg/mL for integrations the of peak area and from 0.05 to 100μg/mL for the peak height, both models with $r^2>0.999$ and $p<0.0001$. The robustness method evaluation was carried out through the variation in the way of preparation of the mobile phase: in test tube, in volumetric bottle or in the HPLC equipment. In all the cases significant differences at the level of 95% were seem which demonstrated that the method is sensitive to the conditions of the mobile phase preparations. The variation coefficient at only 0.25% was found for the mobile phase in volumetric bottle and this way was adopted in the subsequent analyses.

The method presented excellent resolution in relation to β-lapachone which is a decomposition product of laphachol in acid medium with value of 4.53, for peak with retention times and peak widths of 4.33; 0.20 and 5.34; 0.24 minutes, respectively (Figure 2). The detection and quantification limits, respectively, appraised by the signal-to-noise ratio demonstrated through the reference curves obtained, they reveal us values of 0.75 and 2.5ng in an injected volume of 20µL, very lower than that described by Awang et all (1986) [1].

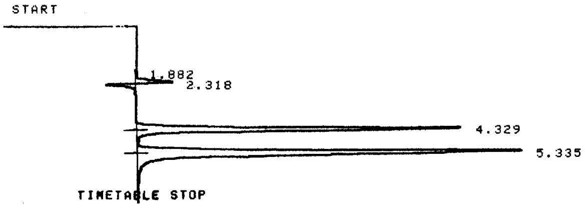

Figure 2 - β-lapachona chromatogram (rt=4,33min) and lapachol (rt=5,34min).

The lapachol solution exposed to the light during a total of 25 hours. The effect of temperature was eliminated by comparison at HPLC profiles obtained for solutions kept in the opaque containers, revealed a zero order degradation kinetics with a specific degradation speed (k) 0.356±0.032µg.mL^{-1}.h^{-1}.

The concomitant action of pH and temperature on the aqueous lapachol solutions gave the respective values of specific degradation speed as shows in the Table I, where it is observed that the pH as well as the temperature directly influence the lapachol degradation. The form of the influence is also dependent on the concentration being zero order decomposition for more concentrated solutions(pH 5.0 with PVA and pH 7.4) and of the first order for the more diluted ones (pH 1.9 and 5.0 without PVA). Lui et all. had already observed decomposition of the lapachol solution and emulsion through spectrophotometric method [4].

Table I - Reaction order and specific degradation speed (µg.mL^{-1}.dia^{-1}) of the lapachol solutions in different pH and temperatures.

pH	Reaction order	Temperature		
		25.2ºC	37.0ºC	50.0ºC
1.9	1	-0.088	-0.113	-0.260
5.0	1	-0.018	--------	-0.030
5.0+PVA	0	-0.299	-0.320	-0.360
7.4	0	-0.434	-0.436	-0.416

The protective action at PVA on the lapachol by showing its degradation rate by a factor more than 12, seems to take place due to formation of hydrogen bridges between the hydroxyls of the alcohol and the carbonyls and hydroxyl of the lapachol. This effect was also held responsible by the increase of the solubility of the lapachol in aqueous solution containing PVA.

Conclusions

Considering the abovermentioned results we conclude that:

- The presented analytical methodology, validated in terms of repeatability, reproducibility, sensibility and linearity, can be used in qualitative analyses for of the lapachol and its preparations;
- considering the low quantification limit, as well as the resolution obtained front to one of its degradation products, the methodology can be used in the evaluation of the degradation of the lapachol and in its pharmaceutical forms;
- The lapachol is sensitive to action of the light and is degraded on a zero order reaction, i.e. which has the rate independent of the initial concentration, for the solutions within the studied interval of 25 hours;
- Lapachol was shown to be sensitive to the pH, being influenced more if it is more ionised in the aqueous solutions; the degradation rate increased with pH;
- The temperature also influenced in a direct way the rate of degradation of the lapachol solutions;
- The interaction of PVA facilitated of the lapachol in the aqueous medium and also accelerated its rate of degradation by the effects of pH and temperature.

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AM-16 DETERMINATION OF LAPACHOL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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Introduction

During the past 30 years, an increasingly intensively search has been under way for chemicals with ant tumor activity. Plants are a particularly interesting source of such substances, since they provide chemicals with novel structures not likely to be synthesized in the laboratory. Thus far many plant products have been found to be active in animal tumor systems, and a few are also effective inhibitors of the growth of some human tumors. Lapachol is a typical example. It is the 2-hydroxy-3-(3-methyl-2-butenil)-1,4-naphtoquinone, is a yellow crystalline material derived of certain Asian and South American bignoniaceous plants. In Brazil, the plant is a tree popularly known as "*ipê-roxo*" or "*ipê*". Several activities have been reported for lapachol including antitumor [1], antibacterial, antitrypanosomal, antimalarial, antiinflamatory [2], analgesic [3] and ulcerogenic effects [4].

The Pharmaceutical Laboratory of Pernambuco State - LAFEPE® (Recife - Brazil), is the only producer of the medicine in the country in the pharmaceutical form of capsules of 250 mg. The raw material was gotten by the Antibiotics Departmen of Federal University of Pernambuco - UFPE - (Recife, Brazil) extracted from heartwood of tropical plants deriving of the region north and northeast of the Brazil.

The current good manufacturing practice require that analytical methods which are used assessing compliance of pharmaceutical products with established specifications, must meet proper standards of accuracy and reliability. Analytical methods for evaluating lapachol in pharmaceuticals using high-performance liquid chromatography (HPLC) have not published. Other methods, as spectrophotometry [1] and chromatography of thin layer, had been developed for evaluation of the stability of formulations of lapachol, having itself shown inadequate for quantification of the active substance.

This paper describes an isocratic reversed phase high-performance liquid chromatographic (HPLC) method to determine concentrations in pharmaceutical forms. This procedure was evaluated using product LAFEPE LAPACHOL® in capsules of 250 mg.

Experimental

• Chemicals

Organic solvent for mobile phase preparation includes HPLC grade methanol omni-solv MX 0488-1 MERCK and purified water gotten of MILLI Q-PLUS. Lapachol for the assay were gotten by the Antibiotic Department of Pernambuco Federal University (Recife, Brazil) and for the standard from SIGMA® Chemical company (USA).

• Instrumentation and Chromatographic conditions

A Costametric® model 3200 Fluid Metering pump (Thermo Separation products, USA) was set at flow rate of 1mL/min. Samples were injected manually using a 20 µL loop. The analytical column was a Merck Lichrocart 125-4 LICHROSPHER 100 RP-18 (100 × 8 mm I.D., 5µm particles) (Water, Milford, MA, USA).

A Spectra System UV 100 detector (Theme Separation Products, USA) was set at a wavelength of 266 nm. Data were using an integrator model SP 4600 (Spectra Physics, USA).

• Mobile phase preparation

The mobile phase was a mixture (v/v) of 90% methanol and 10% purified water. The mobile phase was filtered and vacuum degassed using a 0.45 µm nylon membrane.

• Standard preparation

Dissolve 5 mg of an accurately weighed quantity of Sigma® Lapachol in methanol to obtain a stock solution having a known concentration of about 10 µg/mL and mix per 15 minutes and filter. The similar procedure was applied to obtain concentrations of 5.0, 7.5, 12.5 and 15.0 μg/mL gotten from initial amounts equivalents of standard.

- **Assay preparation**

Transfer an accurately weighed quantity of about 40% of the average weight of the capsules of lapachol of 250 mg to a 50 mL volumetric flask, add 50 mL of methanol to dissolve the specimen, mix per 30 minutes and filter. Dilute with methanol up to obtain a stock solution having a known concentration of about 10 μg/mL. The similar procedure were applied to obtain concentrations of 5.0, 7.5, 12.5 and 15.0 μg/mL gotten from initial amounts of capsules of LAFEPE LAPACHOL®.

Results and discussion

Experiments were performed to establish the linearity, accuracy, precision, specify of the method. The linearity of an analytical method is its ability to elicit test results that are directly, or by a well defined mathematical transformation, proportional to the concetration of analyte in samples within a given range. The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogeneous sample [5].

The chromatograms indicate a retention time for lapachol of 1.8 min. The linearity was verified from 5.0 to 15.0 μg/mL, with intervals of 2.5 μg/mL. The regression equation and correlation coefficient (R) between the peak area (y) and the concentration (x) of lapachol were y=170738x - 350995 and R=0.9998.

The accuracy and precision of the method was verified evaluating the specificity of the method in the presence of the excipients starch, lactose and talc and by repeatability of eight determinations at 50, 100 and 150% of test concentrations, that is, 5, 10 and 15 μg/mL; the results to intra day and inter coefficients of variation are presented in Table 1.

No interferences of the inactive ingredients was detected in the specificity test.

Table 1 –Accuracy and precision of the Method

Standard μg/mL (n=8)	Assay - μg/mL (Mean ± S.D.)	Range	CV (%)
Intra day			
5	4.91 ± 0.09	4.71 - 5.23	1.8
10	10.1 ± 0.16	9.82 - 10.33	1.6
15	14.89 ± 0.30	14.48 - 15.27	2.0
Inter day			
5	4.87 ± 0.05	4.80 - 5.02	1.0
10	9.91 ± 0.11	9.89 - 10.0	1.1
15	15.01 ± 0.26	14.73 - 15.30	1.7

Conclusion

In conclusion, our results indicate that determination of lapachol in pharmaceutical forms by HPLC is a simple, rapid, inexpensive and accurate method that may be used for monitoring parameters of quality of this drug in pharmaceutical industry.

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AM-17 CHARACTERIZATION OF β-LAPACHONE AS RAW MATERIAL FOR DEVELOPMENT OF SOLID PHARMACEUTICAL FORMS

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Introduction

β-Lapachone is a semi-synthetic product obtained by acid hydrolysis or thermal reactions from lapachol, a naphtoquinone that can be isolated from the heart of lapacho tree (*Tabebuia avellanedae*) found in South America [1]. It has been demonstrated several pharmacological activities, such as antimalarial, antibacterial and antitrypanosomal. Recent researches have found that β-lapachone inhibits DNA topoisomerase I, and this fact has improved the research on promising anticancer proprieties of β-lapachone [2].

The objective of this study is to make the characterization of β-lapachone as a pharmaceutical active principle for its use in the development of solid pharmaceutical forms. Actually, the qualification of the raw material used in a technological industrial process is one important way to standardize the conditions of manufacturing and assurance the uniformity of the industrial lot. This study can also determine the purity of β-lapachone produced by Antibiotics Department of Federal University of Pernambuco -UFPE in order to certify the reproducibility of its obtaining method.

Experimental Methods

Chemicals: β-Lapachone, the quinone known chemically as (3,4-dihydro-2,2-dimethyl-2*H*-naphtol (1,2-*b*) pyran-5,6-dione) was supplied from Antibiotics Department of Federal University of Pernambuco – UFPE. It was obtained by an acid hydrolysis with sulphuric acid from the lapachol produced there.

Physical and Physical-Chemical Characterization

Aspect and Morphology: Blades of β-lapachone suspended in purified water were observed with an optical microscope KARLCEISS® equipment, with objectives of 10X and 40X.

Solubility: β-lapachone was tested in different polar and organic solvents, as though in some pharmacological excipients for pharmaceutical formulations.

Residual humidity: This was determined by a METTLER LP-300® infrared balance with 1,0g of powder, which was heated on 105ºC, until constant weight.

Melting Point: This was determined by a capillary method with ELECTROTHERMAL 9300® equipment.

Overlaid Sample UV Spectra: The ultra-violet absorbency spectra of a β-lapachone 10^{-6}g/L solution in methanol, was done to know the absorbency wavelengths.

RMN: The nuclear magnetic resonance spectra was determined to identify the structure of the powder with a UNITY Plus – VARIAN® at 300 MH_z using $CDCl_3$ as solvent.

Thermal analysis: The thermo analytical methods can measure changes of a physical and chemical property, like function of temperature [3]. The thermal analysis by Differential Scanning Calorimeter (DSC) was realized with a mass of 222 mg of β-lapachone powder, in a temperature range between 25-300ºC by an N_2 atmosphere at 50mL/min and a heating rate of 7.5ºC/min.

Pharmacotechnical Characterization [4]

Determination of Draining Time: This assay consisted in marking with a stopwatch the time of passing of 50g of β-lapachone powder through a standatized funnel.

Determination of Rest Angle: This was determined by the measurement of the ray and height formed from the cone because of the fall of powder from 20 cm of height.

Determination of Apparent and Compacted Volume: This essay was realized by putting the β-lapachone in a PYREX® graduated test tube of 250 mL and fixed in an automatic compactor, developed in the laboratory. The volume occupied by 50g of powder was read before and after 1250 beats from a 3 mm of height.

Determination of Apparent and Compacted Density: This was obtained from the relation between 50g of β-lapachone and the volume occupied by powder before and after compaction.

Determination of Index of HAUSNER and Index of CARR: These are two different indexes to express proprieties of the active principle in connection with pharmaceutical technological process such as compression and filling of capsules.

Results and Discussion

Physical and Physical-Chemical Characterization

Aspect and Morphology: β-lapachone can be described as an orange powder, slightly divided, in which the analysis at microscope showed needle crystals of a heterogeneous size.

Solubility: β-lapachone has a lower solubility in water, even at 80°C, but at alkaline pH, this solubility was faintly increased. In ethanol, it was completely soluble, as though in methanol, chloroform, ether, dichloromethane, ethylic ether, dimethyl formamide and benzene. In N-hexane, buthanol and hydro alcoholic solution 50%, β-lapachone was partly soluble. It shows that β-lapachone was soluble at organic solvents with middle polarity.

Residual humidity: Usually a powder must present a residual humidity less than 8%. The humidity of β-lapachone was only 0,3%, what means that its conservation while dry will not have problems due alterations by water.

Melting Point: It was founded that β-lapachone has a 136-138ºC M.P. The quickly and completely melting in a defined point instead of a range indicates an elevated purity of the powder.

Overlaid Sample UV Spectra: The ultra-violet absorbency spectrum of β-lapachone has showed three absorption peaks, at 215, 256 and 264 nm wavelength. The principal was at 256 nm, because it most clear.

RMN: The results of the chemical dislocation were RMN^{1}H: 8,03-7,49 (4H, H_{arom}), 2,55 (t, 2H, CH_2), 1,84 (t, 2H, CH_2), 1,45 (s, 6H, $2CH_3$). They have permitted to identify and to characterize the structure of the β-lapachone.

Thermal analysis: The DSC analysis has showed two-phase transitions, the first one was recognized as β-lapachone at 130ºC and the second one was at 141ºC and was indicate the presence of impurities or secondary products of synthesis. It was possible to appraise the purity of β-lapachone powder produced by Antibiotics Department in 95%.

Pharmacotechnical Characterization

All results are referent of an average of three determinations.

Determination of Draining Time: The β-lapachone powder had very difficult to drain through the hole of the funnel, even with some manual beats, because it stuck on glass walls. The draining time was 6 minutes and when this is more than 10 seconds, it is considerate infinitive. This date shows that β-lapachone powder does not have good fluidity.

Determination of Rest Angle: The result founded for tangent between height and ray resulted in an angle of 42º, which was more than 30º what shows that β-lapachone has a very difficult run off.

Determination of Apparent and Compacted Volume: The initial volume occupied by 50g of powder was 124 mL and after compaction, it was reduced in 26 mL. This value, above 20 mL, indicates that β-lapachone cannot meet appropriately empty chambers, as capsules.

Determination of Apparent and Compacted Density: The apparent density of β-lapachone powder was 0,40 g/mL (Dap) and the volume occupied by powder after compaction was 0,51 g/mL (Dc).

Determination of Index of HAUSNER and Index of CARR: $I_{HAUSNER}$ is defined as Dc/Dap and was calculated as 1,275 and I_{CARR} is defined as (Dc – Dap)*100/Dc and was calculated as 22%.

Conclusion

Due to β-lapachone is a chemically defined powder, the results from physical and physical-chemistry characterization evaluated together, allow to identify the β-lapachone and to conclude that the obtaining method used for its synthesis supply a product with an elevated purity grade, what was confirmed by the thermal analysis (DSC).

The results of evaluation of fluidity, rest angle and draining time, are coherent and they are indicative that it will be necessary to do some pharmacotechnical corrections at rheological parameters during the pre-formulations studies for production of tablets and capsules of β-lapachone.

Therefore, the results of this appraisal allow qualifying the β-lapachone produced by Antibiotics Department of UFPE, as a raw material of high quality. They will permit build the letter of identity of β-lapachone powder and this has a great importance because it refers to a new active principle. Once it is finished, the letter of identity of β-lapachone powder will allow its qualification and standardization, as well as guaranteeing its quality as an industrial raw material.

These results are the preliminary steps for the development of β-lapachone formulations and they are helpful in the development of solid pharmaceutical forms using new concepts of Pharmaceutical Technology.

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AM-18 PROPRANOLOL: VALIDATION OF THE ANALYTICAL METHODOLOGY AND PHARMACEUTIC EQUIVALENCE

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Introduction

Propranolol is a antianginal, antiarrhythmic, antihypertensive, antitimigraine drug, and beta blocker. It's a synthetic beta-adrenergic receptor blocking agent chemically described as 1-(Isopropylamino)-3-(1-naphthyloxy)-2-propanol hydrochloride and available as 10 mg, 20 mg, 40 mg, 60 mg, and 80 mg tablets for oral administration and as a 1 mg/ml sterile injectable solution for intravenous administration. INDERAL®, produced for ASTRA ZENECA is the innovator and reference product in Brazil. With the advent of the law of generic medicines [1], the products to be recognized as such, must: contain the same active ingredients as the innovator drug (inactive ingredients may vary), be identical in strength, dosage form, and route of administration, have the same use indications, be pharmaceutical equivalents, that is, meet the same batch requirements for identity, strength, purity, and quality, be bioequivalent and be manufactured under the same strict standards of good manufacturing practice regulations required for innovator products.

The Pharmaceutical Laboratory of the Pernambuco - LAFEPE® (Recife-PE, Brazil) manufactures Propranolol, since 1996, in 40 mg tablets, and now it intends to transform it into a generic medicine. For in such a way, the procedures of validation of the process and the analytical methodology are being justifd for the fulfilment in the specific legislation [2].

The analytical procedures in the U.S. Pharmacopeia/National Formulary (USP/NF) are those legally recognized as the regulatory analytical procedures for compendial items. The USP 24 [3] foresees HPLC for the analytical procedure of the propranolol tablets. Alternatives analytical procedures can be used since that show perfomance equal or better than the regulatory analytical procedure, that is, the validation of the methodology is required.

The objective of this work is to present the study of the validation of an alternative analytical procedure to propranolol tablets 40 mg and the results of pharmaceutical equivalence of the LAFEPE product in comparison with the product reference INDERAL®.

Experimental

The methodology used in this work to quantify propanolol was spectrophotometry with UV detector in 289nm, using as solvent extractor of active principle the acid diluted clorídrico 1%, in the concentration of 0,02mg/mL.

Experiments were performed to establish the accuracy, precision, linearity, specify, robustness, and limits of quantification and detection of the method.

The accuracy of an analytical method should be established across its range. Here, It was developed by addition of propranolol in concentrations of 50, 100 and 150% to the placebo tablet [3].

Tthe precision was determined in the repetitiveness levels, intermediate precision and reproducibility contemplating the error between tests, analysis, days and laboratories respectively [3].

The linearity was performed from the average of 3 curves and evaluated by the correlation coefficient obtained in the concentrations of 5, 10, 30, 50 e 60 μg/ml. [3].

The specificity was performed fom placebo tablets was prepared contend only the inactive ingredients of the LAFEPE product: talc, manitol, corn starch, polivynilpirrolidone, magnesium stearate [3].

The robustness was carried through from modifications in the parameters of extraction time and concentration of solvent HCl 1% in the variations of 0.7, 1.0 and 1.3%.

Pharmaceutic equivalence was determined by the comparative analysis of the features physico-chemicals and dissolution profile of the tablets LAFEPE and INDERAL. Tests of hardness, average weight, friability, disintegration, had been carried through in accordance with the methodologies and specifications foreseen in the Brazilian Pharmacopoeia [4] For the parameters of concentration, dissolution and uniformity of content followed the specifications of the American Pharmacopoeia, average of six tablets [3]. The dissolution profiles of LAFEPE Propranolol tablets and INDERAL were determined using basket method at 50 rpm

Results and discussion

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The results indicate that the accuracy of the method he was 97,50% for concentration of 50%, and 100% for the concentrations of 100% and 150%.

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogeneous sample. In the present case, the precision tests had not presented coefficient of bigger variation that 0,45% for repeatability and intermediate precision.

The linearity of an analytical method is its ability to elicit test results thar are directly, or by a well defined mathematical transformation, proportional to the concetration of analyte in samples within a given range. The results of the linearity tests had presented straight regression equation and correlation coefficient between samples absorbance and the concetrations of propranolol were $y = 19.381x-2.0594$; $r=0,99999$.

The specificity is defined as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such excipients. Here, placebo tablets was prepared excipientes. No interference of the inactive ingredients was detected in the specificity test.

The robustness is a measure of the method capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The variations in the parameters for evaluation of the robustness of the method, had indicated that these had not been significant and they had not modified the performance of the same.

Table 1 summarizes the results of the physico-chemicals tests carried through in the two tablets and is verified that the quality LAFEPE product is comparable to the of reference product.

The results of the dissolution profile is shown in the Figure 1. It can be observed that product LAFEPE reaches the maximum concentration more quickly than the INDERAL in function of the biggest disintegration time of this last one.

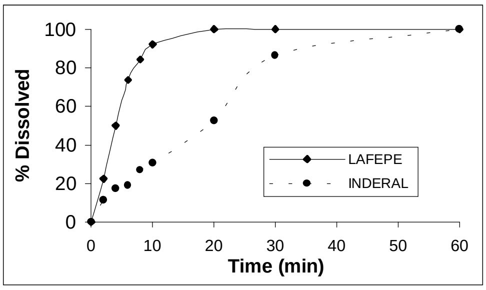

Figure 1 - Dissolution profile

Conclusion

Our results indicate that determination of Propranolol for this method is simple, fast, inexpensive and accurate, and that may be used, surely, in the routine of the pharmaceutical industry. Tablets LAFEPE had demonstrated to physico-chemical quality similar to tablets of reference beyond bigger speed dissolution (Figure 1). With it, could be proven the excellent performance of LAFEPE product and be guaranteed that the same it is pharmaceutical equivalent of the medicine of reference - INDERAL - produced by laboratory ASTRA ZENECA.

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Table 1 Results of the Physico-Chemicals tests - LAFEPE Propranolol® and INDERAL®

PARAMETERS	SPECIFICATIONS	LAFEPE - (RSD%)	INDERAL (RSD(%)
Friability (%)	Max. 2 [4]	0,26	0,10
Average weight (mg)	145 ± 7,5 [4]	152,3 - (3,25)	200,0 - (1,27)
Desintegration (min)	Max. 30 [4]	5	8
Dissolution (%)	Min 75% in 30 minutes [3]	109,89 - (5,34)	93,93 - (11,62)
Hardness (Kgf/cm^2)	Min. 4,0 [4]	6,7	5,1
Concentration (%)	90 a 110 [3]	98,25	99,31
Contents Uniformity	85 a 115 [3]	104,18 - (1,8)	100,93 - (1,57)

AM-19 DETERMINATION OF GATIFLOXACIN IN TABLETS BY NONAQUEOUS TITRATION WITH PERCHLORIC ACID

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Introduction

Gatifloxacin (GTFX) ((±) – 1 – cycloproppyl – 6–fluoro-1,4-dihydro-8-methoxy-7-(*cis*-3,5-dimethyl - 1-piperazinyl)-4-oxo-3-quinoline carboxylic acid), an oral fluoroquinolone antibacterial agent, is active against most aerobic Gram-positive and Gram-negative organisms and demonstrates moderate activity against anaerobes and Mycobacteria, which the quinolones in general have low activity [3]. Although GTFX has been studied in terms of therapeutic activity [4,5,7] and commercialized, there is no official pharmacopoeial monograph on its quantification in raw material and tablets. Few reports about analytical methods in biological fluids are available in the literature such as UV-spectrophotometry, HPLC [1,6]. Nonaqueous titration is the most common titrimetric procedure used in pharmacopeial assay. Owing to the widespread use of nonaqueous titration, it is important that reproducible methods are developed and that these are thoroughly validated.

Experimental methods

- **Materials**

GTFX reference substance (GTFX-RS) (99.1% purity, MW 275) and GTFX tablets (GTFX-TB) were kindly supplied by Brystol-Myers Squibb Brasil S. A. The GTFX tablets (Tequin™) were claimed to contain 400 mg of active drug. All other chemicals were analytical grade. Glacial acetic acid (Merck); perchloric acid 0.1 *M*; crystal violet 0.1% (in acetic acid) as indicator.

- **Tablets of gatifloxacin**

In order to determine the average weight, 20 tablets were accurately weighted. Twenty tables were crushed and dried at 105°C by 2 hours. In a 250 mL conical flask containing a magnetic bar, 602,80 mg of accurately weighted GATX tablets were dissolved in 40 mL of glacial acetic acid and 5 drops of indicator were added. In order to determine the percentage, these solutions were titrated with perchloric acid 0.1 *M* until colour change which indicated the final point was reached (blue). A blank determination was performed in the same manner to make any necessary correction. Precision of titration method was based on 5 determination.

1 mL 0.1 *M* perchloric acid ≡ 37.54 mg of $C_{19}H_{21}O_4N_3F$

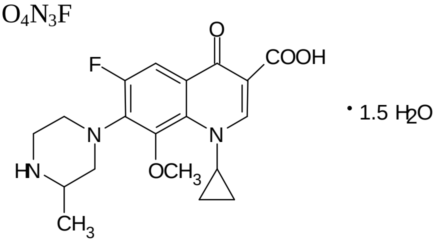

Figure 1– Chemical strucutre of gatifloxacin.

- **Recovery Test**

The accuracy was determined by adding amounts of the reference substance to the samples at the beginning of this procedure [7,8].

The recoveries were determined by adding known amounts of GTFX-RS (10.1; 20.3 and 39.7 mg) to the samples at the beginning of the procedure. The GTFX-RS and GTFX-TB were dried at 105°C for 2 h. The equivalent of average weight GTFX-TB was spiked with amounts of GTFX-RS and was dissolved in 40 mL of glacial acetic acid. Finally, this sample was tritated with perchloric acid 0.1 *M* to quantify as described above.

- **Validation**

The accuracy and precision of this method were determined for intra- and inter-day on three different days. The precision was expressed as the percent coefficient of variation. The statistical analysis employed mean, standard deviation, coefficient of variation [7].

Results and discussion

The majority of all medicinal agents are weak salt or basis and can be assayed in nonaqueous medium. The British Pharmacopoeia [2] recommends a titrimetric assay for nalidixic acid, which is dissolved in dichloromethane and isopropanol, using ethanolic sodium hydroxide as the titrant. The detection of the end point is accomplished potentiometrically. Norfloxacin, a first generation quinolone, is titrated in glacial acetic acid with perchloric acid and detecting potentiometrically the end point [7]. The results obtained through the titrimetric analysis with GTFX-TB using perchloric acid show 100.45% of GTFX and coefficient of variation 0.64%.

According to the literature [7, 8], the accuracy of the method can be measure by the recovery rate which was obtained by comparing the experimental results to the calculated theoretical concentrations.

The results of recovery test of GTFX in tablets using the volumetric determination with perchloric acid 0.1 *M* are in agreement with spiked amount of reference substance.

The reaction between GTFX and a nonaqueous medium and glacial acetic acid is an acid-base reaction where the strong acid can donate a proton to nitrogen from piperazinyl ring. The proposed analytical method in a nonaqueous medium shows to be an important tool for precision and accuracy in the quantification of GTFX raw material and tablets.

The nonaqueous titration can be applied routinely, because it does not require high cost equipment. There was no evidence of interference from excipients in the tablets analysed. The standard deviation and the C.V.% were found to be less than 1.0%, indicating good repeatability of the nonaqueous titration. This volumetric method proposed is simple, rapid and inexpensive and can therefore be applied to the determination of GTFX raw material and tablets. Method validation yielded good results and included precision and accuracy.

Table 1– Data obtained from tablets analysis by nonaqueous titration

Theoretical (mg)	Experimental (mg) ± S.D.	Purity (%)	C.V.(%)
400	401.8 ± 0.025	100.45	0.64

Conclusion

This volumetric method proposed is simple, rapid and inexpensive and can therefore be applied to the determination of gatifloxacin raw material and tablets. Method validation yielded good results and included precision and accuracy. It was also found that the excipients in the commercial tablet preparation did not interfere with the assay.

Acknowledgements

We thank the Brystol-Myers Squibb Brasil S. A. for providing gatifloxacin reference substance and gatifloxacin tablets. This work was partially supported by PACD-FCFAr-UNESP.

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AM-20 HPLC ANALYSIS OF THE FLAVONOIDS IN EXTRACTIVE PREPARATIONS FROM *Achyrocline satureioides* (LAM.) D.C. – COMPOSITAE

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Introduction

The *Achyrocline satureioides* (Lam.) D.C., Compositae, plant known as "marcela" or "macela", is widely used in Brazilian folk medicine, as digestive, anti-spasmodic, anti-inflammatory, hypoglicemic, and to reduce blood cholesterol levels [6]. Several reports on its composition have demonstrated that the flavonoids quercetin, 3-O-methylquercetin [3] and luteolin [6] are the main constituents of its ethanol extracts.

High performance liquid chromatographic (HPLC), has been chosen for flavonoid quantification in vegetable raw materials and extractive preparations. For *A. satureoides*, HPLC methods has been reported, where quercetin has been separated from luteolin and 3-O-methylquercetin. However the separation of two last ones has not been reached.

For validation of analytical methods the guidelines of the 4 International Conference of Harmonization (ICH 4, 1997) as USP XXIV (2000), have recommended the accomplishment of tests of accuracy, precision, specificity, linearity, work strip and robustness of the method. The type of method and its respective use determines what parameters should be evaluated.

Although an expressive number of reports concerning to the validation of analytical methods of drugs, has been found and isolated substances [1], the works on validation of methodologies of control of quality of preparations obtained from medicinal herbs are scarce.

In *A. satureioides* preparations, although the flavonoids are not the only constituents responsible by the pharmacological activity, these compounds have been employed as reference.

The present work was design to develop an HPLC method in view to separate the three main flavonoids of the extractive *A. satureoides* preparations, as well as to analyze the linearity, repeatability and intermediary precision of the method.

Experimental methods

- **Extractive preparations**

The aqueous extractive solution (EAQ) was obtained by decoction, in a plant:solvent ratio of 0.75:10. Two other extractive solutions denominated, E40 and E80, were respectively prepared by maceration, in ethanol 40 % and 80% (v/v).

- **Chemicals**

Quercetin (Merck–art.7546) and Luteolin (Sigma–L9283)

- **Instrumentation**

HPLC analysis was performed using a pump Waters 510, equipped with automatic controller of flow Waters 600, manual injector Rheodyne 7125, Waters 486 UV detector and a Waters 746 plotter.

- **Separation system**

HPLC analysis was performed using a column Shimadzu (CLC-ODS (M)) RP-18 (250 mm x 4 mm I.D.). The mobile phase consisted of two solvents: methanol and solution of H_3PO_4 1% (m/v), in the proportion 53:47 (v/v), flow of 0,6 ml/min and detection of 362 nm.

- **Reference substances**

Quercetin and luteolin were dissolved in methanol and water (53:47 v/v), giving final concentrations of 1.0; 1.5; 2.0; 2.5; 5.0 and 10 μg/ml.

- **Preparation of samples**

Aliquots of 0.5, 1.0, 2.0, 3.0 and 4.0 ml of extractive solutions were diluted in mobile phase to 20 ml, obtaining the final concentrations of 2.5, 5.0, 10, 15 and 20 μl/ml. The linearity of the curves obtained from EAQ, E40 and E80 were evaluated.

- **Determination of the repeatability and of the intermediary precision**

For evaluation of the repeatability, the standard deviation and variation coefficient corresponding to a number of five injections were considered, and for evaluation of the intermediary precision were considered different days of analysis (n=3). This analysis only applied to the extractive solution E80.

Results and discussion

The regression coefficients (r^2) obtained for the standard calibration curves of quercetin and luteolin, were respectively, of 0.99917 and 0.9992, demonstrating the linearity of the method for the concentration range of 1.0 to 10µg/ml.

The several reports regarding the HPLC analysis of flavonoids from *A. satureioides* has shown a very resolved peak of quercetin, while they didn't have success in the separation of the flavonoids luteolin and 3-O-methylquercetin. For this reason these two later have been dosed together, expressed values in luteolin concentration [2,4,5]. The chromatography system employed in this study demonstrated, at the first time, the separation of the flavonoids luteolin and 3-O-methylquercetina, which present retention times of, approximately, 28 and 30 minutes, respectively, as well as good resolution, narrow peaks and reproducible areas.

In view to evaluate the interference of the other constituents, the linearities of the curves obtained from EAQ, E40 and E80 in the concentration of 2.5, 5.0, 10, 15 and 20 µl/ml were analyzed. Unfortunately, we had 3-O-methylquercetin in amount enough only to identify the substance and not to quantify it.

The regression analysis of the calibration curves obtained by HPLC of the EAQ and E80, respectively, linear regression coefficients (r^2) of 0.99894 and 0.99741, for the peak area of quercetin, and 0.99403 and 0.99729, respectively, for referring areas to the luteolin. The results demonstrated that linearity for the extractive solutions EAQ and E80 was very satisfactory, once they are complex samples.

The E40 presented deviation of the linearity in the point of the curve corresponding to the concentration 15 µl/ml, resulting in linear regression coefficients of 0.90835 and 0.89527, for to peak areas to the peaks of quercetin and luteolin, respectively.

The comparison of quercetin and luteolin concentrations in EAQ, E40 and E80 demonstrated these flavonoids were better extracted by the solvent of smaller polarity. The repeatability of the HPLC analysis of E80 was also demonstrated, with percent variation coefficient of 4.0 % for E80 in the concentration of 10 µl/ml. The intermediary precision of this sample showed a percent variation coefficient of the 6.23 %.

Conclusions

The HPLC method proposed in this report allow to separate the three main flavonoids present in *A. satureoides.*

The method showed linearity in the analysis of the quercetin, luteolin, extractive solutions EAQ and E80, and don’t showed linearity for extractive solution E40.

The HPLC method showed repeatability and intermediary precision in the analysis of the extractive solution E80, 10 µl/ml concentration.

Acknowledgements

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AM-21 APPLICATION OF THE EXTRACTION MICROWAVES FOR ANALYSIS OF THE ALKALOIDS OF *Cissampelos sympodialis* (Menispermaceae)

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Introduction

The search for modern extraction techniques applied to the control of quality of medicinal plants appears as a demand of pharmaceutical industries, as well as of environmental agencies (VILEGAS, 1997; PINTO, 1998; ARAGÃO, 2000).

Sample preparation using microwave oven is very scarce in the literature (ARRUDA, 1997). Microwaves appears as a powerful alternative technique to classical extraction methods, due to advantages such as shorter extraction time and smaller amounts of solvents.

Alkaloids bisbenzylisoquinoline have a great importance for the pharmacological activity of several medicinal plants. Including those of *Cissampelos symposialis.* A hot infusion of the leaves of *Cissampelos sympodialis* Eichl. (Menispermaceae), a plant extensively used in Northeast Brazil for asthma, bronchitis, influenza, and rheumatism, among other conditions (CORTES, 1992). Warifteine, a bisbenzylisoquinoline alkaloid, which is one of the major (approx. 1.7 %, w/v) alkaloid constituents of the aqueous fraction was also found to present antispasmolytic activity (CORTES, 1995; FREITAS, 1996). The present report shows the results of microwave extraction for the extraction of alkaloids from 'milona' leaves (*Cissampelos sympodialis* Eichl).

Experimental methods

• Plant Material

The leaves of *Cissampelos sympodialis* Eichl (Menispermaceae) were colleted from plants grown in the botanical garden of the University and voucher specimens were deposited (code, Agra 1456) with the Botany department. The leaves were dried at 40 °C in an oven and then pulverized. Powdered material was stored protected from light and moisture.

• Extraction of plant material

The microwave system used in present work was Provecto system, model DGT 100. The experimental conditions utilized in microwave oven were:

EM1: 1 g of the dry leaf of 'milona' was submitted the extraction in a microwave oven with 30 mL of ethanol 80°GL and submitted to the following programs: 1) extraction time, 4 minutes; potency, 500 W; 2) extraction time, 5 minutes; potency, 200 W.

EM2: 1 g of the dry leaf of 'milona' was submitted the extraction in an microwave oven with 30 mL ethanol:water (1:1) and submitted to the following programs: 1) extraction time, 4 minutes; potency, 500 W; 2) extraction time, 5 minutes; potency, 200 W.

EM3: : 1 g of the dry leaf of 'milona' was submitted the extraction in an microwave oven with 17 mL water: chloroform:HCl at 5% (15:1:1) and submitted to the following programs:
1) extraction time,4 minutes; potency, 500 W;
2) extraction time, 8 minutes; potency 500 W;
3) extraction time, 12 minutes; potency, 500 W;
4) extraction time, 5 minutes; potency, 200 W;
5) extraction time, 10 minutes; potency, 200 W;
6) extraction time, 15 minutes; potency 200 W.

The several extracts EM1, EM2 and EM3 were acidified and the alkaloids presence monitored with solution of Dragendorf.

Results and discussion

In this work an extraction method was developed through microwave oven for the alkaloids analysis of the leaves of *Cissampelos sympodialis.*

• Choice of the solvent, extraction time and potency

Among the utilized solvent, **EM2**, ethanol:water (1:1) showed the best extraction. The best extraction was achieved using extraction time, 5 min. and potency, 200 W (table 1).

The samples **EM3** were submitted to a extraction potency of 500 W suffered degradation due to high energy involved in the extraction process, not being efficient for the alkaloids extraction of the milona. The acidified samples did not extract the alkaloids. The increase in the time of extraction did not alter the result (table 1).

Table 1 - Samples obtained in microwave oven in different conditions of extractions.

Sample	Program (extraction time; potency)	alkaloid test*
EM1	4 min.; 500 W	-
	5 min.; 200 W	+
EM2	4 min. ;500 W	-
	5 min.; 200 W	+ + +
EM3	4 min.; 500 W	-
	8 min.; 500 W	-
	12 min.; 500 W	-
	5 min.; 200 W	-
	10 min.; 200 W	-
	15 min.; 200 W	-

*(-) negative; (+) not very positive; (+ + +) very positive

Conclusions

The results presented in this work indicate that microwave extraction is a valuable alternative technique to conventional methods for the extraction of the alkaloids from “milona” (*Cissampelos sympodialis* Eichl). Besides the advantages inherent to the microwave oven (e.g., economy of solvents and time), microwave can be a tool for the analysis of reduced amounts of medicinal plants.

Acknowledgements

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AM-22 COMPARATIVE STUDY OF THE CHROMATOGRAPHIC PROFILE OF THE SPECIES *Schinus Terebinthifolius* RADDI AND *Astronium Urundeuva* ENGL AS PARAMETER OF QUALITY CONTROL

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Introduction

Schinus terebinthifolius Raddi, "Aroeira da Praia (AP)", and *Astronium urundeuva* Engl, "Aroeira do Sertão (AS)" are often used in the Brazilian Folk Medicine and they can be found in the coast-land and arid region of the Northeast respectively [1]. They are widely used as antiseptic, antiphlogistic, healing and adstringent. These properties have been confirmed in previous works [2, 3, 4]. The bark's decoct has been prescribed in different pharmaceutical dosage forms such as ovule and creams (phytopharmaceutical), as a therapeutic alternative in the treatment of gynecological wounds and escorry, respectivelly

The determination of the chromatographic profile is an efficient laboratorial technique, for evaluation of the authenticity of vegetable drugs as well as the identification and isolating their active principles.

Motivated by phytopharmaceutical quality control requirements, the present study aims the determination of the chromatographic profiles comparing the two species *Schinus terebinthifolius* Raddi and *Astronium urundeuva* Engl through Thin Layer Chromatography (TLC) using differents detection reagent.

Experimental methods

- **Materials**

Schinus terebinthifolius Raddi, and *Astronium urundeuva* Engl, barks, alcohol 70° GL, sulfuric acid solution at 5%, glass plates coated with cellulose 0.50 mm layers of cellulose (MERCK), gallic acid (Sigma), Acetic acid (Qimex), distilled water, Chloroform (Qimex), Methanol (Vetec), n-buthanol (Vetec), $FeCl_3$ 1% in ethanol, Vanillin Chloridric 1% in methanol, Folin-Ciocalteau reactive and Ammonia vapor's.

- **Preparation of hydroalcoholic extracts**

The hydroalcoholic extracts were prepared by maceration (with 1:10 (w/v) plant/solvent ratio), then submitted to the acid hydrolysis using sulfuric acid solution at 5% during 1.5 h under reflux, (conditions previously standardized) [5].

- **Chromatographic profile**

TLC was performed on 20 x 20 cm TLC glass plates coated with cellulose 0.50 mm layer. Aliquots of 4μL of raw extract and hydrolyzed fractions were applied on plates and submitted at different chromatographic systems and specific derivatization reagent for polifenolics (Table 1), using gallic acid as standard. Among the various systems tested those that showed the best results are presented in the table 1.

Table 1: The chromatographic systems

Systems	Mobile phases	Detection reagent
1	Acetic acid/water (30:100, v/v)	UV light λ=254nm and 360nm
		$FeCl_3$ 1% in ethanol
		Folin-Ciocalteau reactive and Ammonia vapor's
2	Chloroform/Methanol/ water (26:14:3 v/v/v)	UV light λ=254nm and 360nm
		$FeCl_3$ 1% in ethanol
3	n-buthanol/acetic acid/water (4:1:2 v/v/v)	Vanillin Chloridric 1% in methanol
		UV light λ=254nm

Results and discussion

The chromatograms for AP and AS showed different profiles, by the presence of characteristic spots with different Rf values (table 2). For the three systems, AP and AS presented a blue spot with the same Rf corresponding to the gallic acid standard, common in both species (table 2 and figures 1 and 3). In contrast, AP exhibited a yellow spot (Rf = 0.15) with system 1 while AS presented both a yellow spot (Rf = 0.11) and a white one (Rf = 0.60) as showed in figure 2. The white spot was also observed for AS with system 2 (Rf = 0.77). So, these systems can be used to establish the difference between the two species through the white spot. The system 3 was not usefull to differentiate AP and AS.

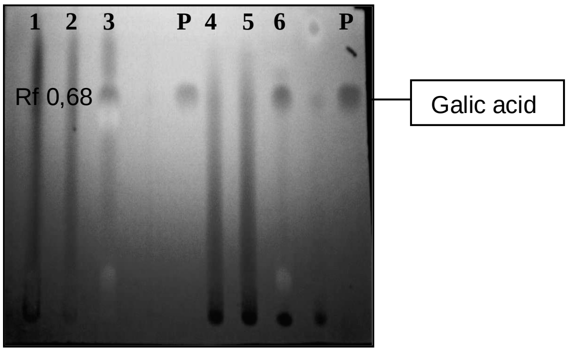

Figure 1 Comparative chromatographic profile of the hydroalcoholic extracts AS and AP (1 and 4), spray dried extracts (AS-2 and AP-5), hydrolysis fractions AS and AP (3 and 6 respectively), gallic acid standard (P) and system 1 with UV light λ=254nm.

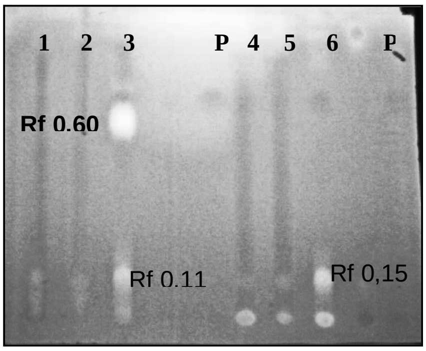

Figure 2 Comparative chromatographic profile of the hydroalcoholic extracts AS and AP (1 and 4), spray dried extracts (AS-2 and AP-5), hydrolysis fractions AS and AP (3 and 6 respectively), gallic acid standard (P) and system 1 with UV light λ=360nm.

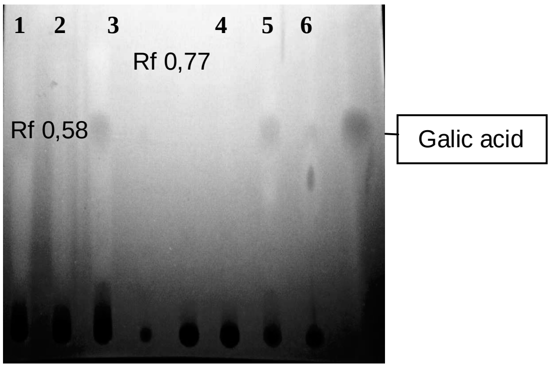

Figure 3 Comparative chromatographic profile of the hydroalcoholic extracts AS and AP (1 and 4), spray dried extracts (AS-2 and AP-5), hydrolysis fractions AS and AP (3 and 6 respectively), gallic acid standard (P), system 2 with UV light λ=240nm.

Table 2: Rf values of spots of the extracts *Schinus terebinthifolius* Raddi (AP) and *Astronium urundeuva* Engl (AS).

Systems	Spots	Rf Values	
		AP	AS
Acetic acid/water (30:100, v/v)	White	-	0.60
	Yellow	0.15	0.11
	Blue	0.68	0.68
Chloroform/Methanol/water (26:14:3 v/v/v)	White	-	0.77
	Blue	0.58	0.58
n-buthanol/ acetic acid /water (4:1:2 v/v/v)	White	-	-
	Blue	0.77	0.77

Conclusion

The results demonstrated that TLC can be used as fast, economic and practice technical to evaluate the different species of Aroeira da Praia (AP) and Aroeira do Sertão (AS). The chromatograms showed that the white spot can be used as standard in the difference between species.

Acknowledgements

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AM-23 PHYSICO-CHEMICAL PARAMETERS AND QUANTITATIVE ANALYSIS OF COUMARIN AND UMBELLIFERONE IN SYRUPS OF *Justicia pectoralis* BY HPLC

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Introduction

"chamba" syrup (*Justicia pectoralis* var. *stenophylla* Leonard, Acanthaceae) prepared from the plant leaves is utilized in public health programs as bronchodilator and antiinflammatory. Studies carried out with leaves from *J. pectoralis* demonstrated the presence of several compounds, including coumarin, umbelliferone, caffeic acid, glycosides, N,N-dimethyl tryptamine (Joseph, 1988; Taveira, 1993; Matos, 1994). Previous works (Lino et al., 1997; Leal et al., 2000) demonstrated that the hidroalcoholic extract, coumarin and umbelliferone from *J. pectoralis* presented antinociceptive, antiinflammatory and bronchodilator activities.

The objective of this work was to report the phytochemical profile and the use of HPLC for the quantitative analysis of the coumarin and umbelliferone, as markers for syrups from *J. pectoralis*.

Experimental Methods

- **Plant material**

The authenticated plant material was collected at the city of Fortaleza, Ceará, Brazil. Voucher specimens (Nos. 16071 and 16079) were deposited at the Prisco Bezerra Herbarium, Department of Biology, Federal university of Ceará, Brazil.

- **Physico-chemical parameters**

The physical-chemical parameters were performed as described by Brazilian Pharmacopoeia 4th edition. It was determined mean volume, density and viscosity. Each determination was carried out in triplicate.

- **Phytochemical profile**

The phytochemical profile was performed as described by Costa (1977). It was determined by identification reactions based on the chemical group to be determined or thin layer chromatography.

- **Chromatographic analysis**

High Performance Liquid Chromatography (HPLC) analyses were performed on Shimadzu CLASS-LC 10, with detection UV (254nm). The column RP-18 (Shimadzu) was 250 mm x 4 mm x 5µm was used. The samples were injected at volume of 20 µL and colunm temperature was 40°C. The mobile phase was acetonitrile:water at 50%, isocratic and flow of the 1mL/min. The results were expressed as means ± SEM.

Quantitative analysis. Estimation of the content of coumarin and umbelliferone in chambá syrup was performed by external calibration. Aliquots (20µL) of concentrations of 0.25; 0.5 and 1.0mg/mL of coumarin and umbelliferone were analysed by HPLC, and the areas under the peaks for each dilution were recorded. Standard curves were drawn by plotting peak mean areas against the injected amount of each standard.

Results and discussion

The physico-chemical parameters of "chambá" syrups, as mean volume, pH, density, viscosity were 102 ± 0.03 mL; 7.16 ± 0.005; 1.2 ± 0,001g/mL; 24.00 ± 0.045 cp (temperature: 24.3°C; torque: 72.2%) respectively.

Table 1 presents the phytochemical profile of the *J. pectoralis* and shows that besides coumarin and umbelliferone other components where detected.

Figure 1 shows a typical chromatogram of coumarin and umbelliferone (1mg/mL) with retention times of 8.6 and 12,2 min respectively. Figure 2 shows the chromatogram of coumarin and umbelliferone found in samples of syrup of *Justicia pectoralis* obtained from hydroalcoholic extract (20%) as intermediate products, analysed in triplicate by HPLC. Concentrations of coumarin and umbelliferone from the syrups were 97.00 ± 0.351 µg/mL and 154.00 ± 0.057 µg/mL respectively.

HPLC with ultra-violet detector showed to be sensitive and reproducible for the analysis of

Table 1
Phytochemical profile of *Justicia pectoralis* (chambá).

Chemical group	chambá
Coumarin	+
Flavonoid	+
Saponin	+
Cardiac glycoside	-
Anthraquinone	-
Tannin	+
Alcaloid	-

Specific reactions for the chemical groups or thin layer chromatography were utilized: +, presence; -, absence.

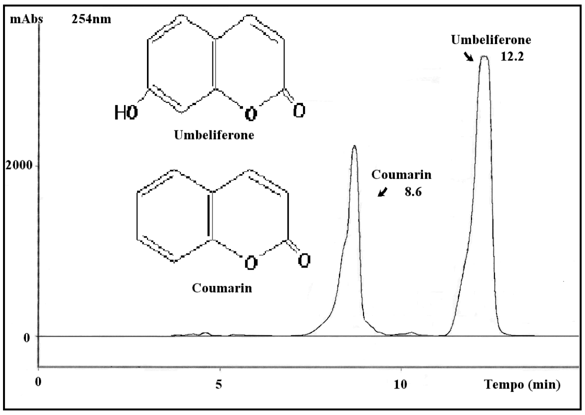

Figure 1. Chromatogram of coumarin and umbelliferone (1mg/mL)

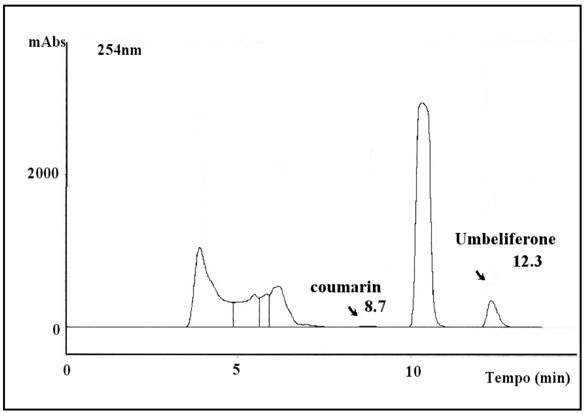

Figure 2. Chromatogram of "chambá" syrup produced from hidroalcoholic extract of the plant.

coumarin and umbelliferone. It may be a useful method considering that Brazilian Pharmacopoeia doesn't describe so far any methodology for constituents in either crude drug or in phytomedicines from "chambá".

Conclusion

This method is suitable for simultaneous determination of coumarin and umbelliferone in syrups of *Justicia pectoralis*, although further studies are necessary to establish methodologies to be followed in the quality control of this phytomedicine.

Acknowledgements

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AM-24 PRESENCE OF BHT IN THE ESSENCIAL OIL OF BRYOPHYTES (SUB CLASS BRIDAES)

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Introduction

Bryophytes, collective term applied to about 22,000 species of small plants that usually grow in moist areas on soil, tree trunks, and rocks. Bryophytes are nonvascular embryo-bearing plants of three plant divisions: the Bryophyta (see Mosses), the Hepatophyta (see Liverwort), and the Anthocerophyta (hornworts). True conductive tissues, such as those in ferns and higher vascular plants, are not found in bryophytes. Although they range in size from microscopic to 30 cm (12 in) in length, the average bryophyte is between 1.2 and 5 cm (0.5 and 2 in) long and varies from green to black to almost colorless [1, 2, 3]. The class of mosses have a central plant body resembling a stem from which tiny leaves radiate and rootlike structures, called rhizoids, extend. The embryo from the sexual plant matures into a small asexual plant that remains attached to and dependent on the sexual plant. The asexual plant produces spores similar to the spores produced by lower plants, and they are spread by wind and other carriers to produce new sexual plants. The sexual organs of bryophytes are multicellular. The bryophytes display an important part on the ecological equilibrium. They are also important on the pollution monitoring and on the extraction of useful substances applied in clinical therapeutic and industries of food, pharmaceutical preparations and cosmetics [4, 5]. On the perspective of substances applied in these industries, BHT (butyl hydroxytoluene) (figure 2) is usually added to foods, pharmaceutical preparations and cosmetics to preserve fat acids. Oxygen will react preferentially with them, instead of oxidizing (and spoiling) the fat [6, 7, 8].

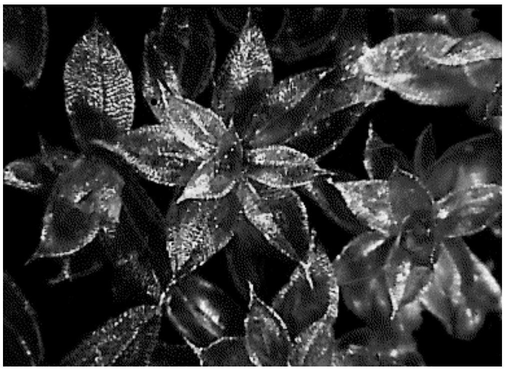

Fig.1 *Funaria flavicans*, sub class Bridae

BHT have bulky hydrocarbon groups connected to a phenol ring, which makes them both fat-soluble and oxidizable, like Vitamin E, a natural fat-soluble antioxidant, having similar structural features. Some studies indicate that it has some antiviral activity and antimicrobial properties [8].

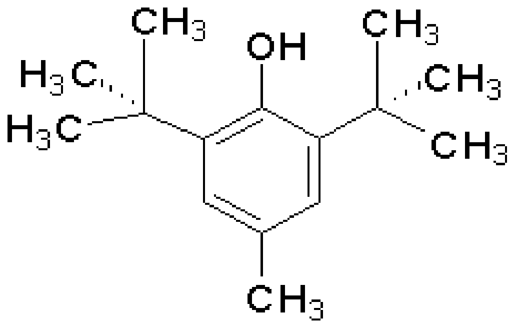

Fig. 2 Chemical structure of BHT

The purpose of this study was the extraction of the essential oil of bryophytes (sub class *Bridaes*) (figure 1) and the chemistry characterization of these components.

Methodology

The extraction of the oil was done by steam pull hydrodestilation on Clevenger apparatus modified by Wasicky on Nitrogen atmosphere and constant temperature of 55°C [9] (figure 3).

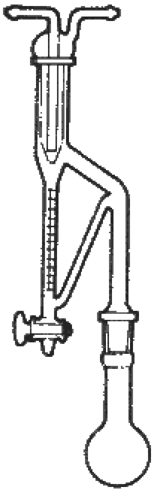

Fig 3 Clevenger apparatus modified by Wasicky

GC/MS-MSD 5890A/5970 (High precision gas chromatography coupled to a mass detector) has been used for this essential oil with Wiley-Windows data system for identify the components. Injected 0.15 µL of essential oil sample on proportion of Split 1:100 with initial temperature of 4°C, partial temperature of 158°C on 10 minutes, heating ramp of 4°C/min. Modified the progression to 6°/min of the heating ramp when the partial temperature was 180°C in 20 minutes [10. 11]. The final temperature was 250°C in running time of 96.67 minutes. The capillary column of methyl silicone PONAS' type with 50 m x 0,2 mm x 0,5µm of liquid film on sewage of 4 ml/min He. Injector's temperature: 250°C, Ionization energy: 70 eV, scanning from 20 to 550 u.

Results

69 components were found into the essential oil. The main found substance was the hydroxitoluene buthylated. It is important as an antioxidant agent of extreme prominence for the food industry and for pharmacotechnic, besides the BHT is the component in major concentration (11.93% v/v). In addition, others components earn quantitative importance like the Fitol (0.59% v/v), Octodecanoic acid (0.67% v/v), Geranial (2.03% v/v), and Hexadecanoic acid (5.46% v/v). It was also observed on this oil the presence of sesquiterpens, oxigened terpens and hydrocarbons.

Conclusion

The concentration of BHT found on this sample is significant (11.93%) and can mean a change of extraction of this antioxidant.

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AM-25 THE USE OF *Lychnophora ericoides* Mart. IN PHYTOTERAPICS: A PHARMACOGNOSTIC CHARACTERIZATION MODEL

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Introduction

The use of medicinal plants plays a key role in world health and preparations consisting of plants or their derivatives as active compounds have been popularized worldwide in the more recent years. On the other hand studies of characterization, standardization, stability, and quality control of many medicinal plants species and herbal preparations have been poorly performed or are not well defined in the literature. However the safety, efficacy and quality control of medicinal plants and phytoterapics agents are important parameters to for the development of phytomedicines.

Lychnophora ericoides Mart. (Asteraceae) is an endemic plant of rupestris regions in Brasil and it is known in the traditional medicine as 'false arnica'. The leaves of *ericoides* are used for external application mainly in the form of tincture or hidroethanolic macerate of the intact leaves, both as an anti-inflammatory agent. Earlier phytochemical investigations of *L. ericoides* reported mainly the presence of sesquiterpene lactones as the compounds responsible for pharmacological action. Some earlier phytochemical studies with Asteraceae species reported sesquiterpene lactones stored in glandular trichomes. These strutures were observed in the leaves of *L. ericoides* after microscopic analysis. Due to the fact that *ericoides* is not present in any pharmacopoeia and based on its current use by the traditional medicine as well as the importance of quality control methods novaday, we decided to perform the characterization of *L. ericoides* and its extractive solutions.

Materials

Leaves of *L. ericoides* (Asteraceae), ethanol, petrol ether, ferric chloride, chloridric acid, chloroform, sodium sulfate, ammonium hydroxide, methanol, n-pentane, ethyl acetate, ethyl ether, formic acid, glacial acetic acid, hexane, methylene chloride, silica gel, samples of sesquiterpene lactones, flavonoids and Dragendorff, Meyer, Zimmermann, Libermann - Buchard reagents.

Methodology

- **Plant collection**

The aerial parts of *L.ericoides* were collected at Ibiraci – Minas Gerais, Brazil, in April 2000 and the material was identified by Prof. João Semir, Institute of Biology – UNICAMP, SP. The leaves were separated by manual process.

- **Preparation of the plant material for determinations**

One part of the leaves was air-dried at 40 ºC and followed to pulverization. For the phytochemical studies powdered leaves were extracted with chloroform followed by a 70% (v/v) hidroethanolic mixture.

- **General determinations**

Phytochemical profile*:* This method has been performed through chemical reactions with the lipophilic and hydrophilic extracts of *L. ericoides* with specifics reagents in order to identify terpenoids, tannins, flavonoids, alkaloids, phenolics compounds, saponins, sesquiterpene lactones, steroids and antocianins. The results were observed by change of color and / or formation of precipitates.

Drying loss and total ash: The drying was determined by the gravimetric method. The powdered sample was heated in an oven (105^{o}C) until constant weight. The obtained residue was expressed in the percentage of drying loss, in reference to the air dried drug. The analysis of total ash was performed according to the description specified in general methods for the analisys of plant drugs. The sample was incinerated at 550 - 600^{o}C until constant weight and the residue obtained was expressed in percentage.

Extractable fractions: It was performed in boiling solvent followed by percolation of the drug in Soxhlet apparatus. The determination was processed with ethanol and diethyl ether until exhaustion. After the solvent had been evaporated and dried, the residue was weighted. The results were expressed by the average of three determinations.

Extractives content: The drug and an amount of water were heated during a certain time period, followed by reconstitution of the initial weight with the same extrative solution at room temperature. The solution was filtered, evaporated and dried at 105^{o} C until constant weight.

Swelling index: This process was performed in a 25 mL graduated cylinder with defined dimensions and the results were available in milliliters occupied by the drug after it has been swollen in aqueous and ethanol liquids (separated) and allowed to stand in specified times**.**

Foam index: After heating the drug in water, the product was filtered to a volumetric recipient and the final volume was completed with the extractive solution. Several dilutions were made in different tubes that have been shaker in vertical movements for specific time periods an then followed to stand. The foam index was observed as the height of foam after the estimated time.

- **Preparation of sample for chromatographic analysis**

One amount of powdered leaves was extracted in a mixture (v/v) of water and methanol (1/1) using a reflux condenser. The filtrate was concentrated under reduced pressure followed by a second extraction with ethyl acetate. The ethyl acetate fraction was dried, submitted to column chromatography and the eluate obtained was dissolved in a mixture (v/v) of methanol -water (1/1), treated with alluminium oxid, centrifuged and filtered. The solvent was removed and the extract obtained was used for analysis by thin layer chromatography (TLC). Its was carried out on precoated plates of kiesegel 60 (Merck). The used solvent system were specific to detect sesquiterpene lactones and flavonoids. Other extraction methods were applied for powdered leaves of this species and then submitted to analysis by TLC.

- **Preparation of the solutions to the use in formulations**

• Superficial material solutions of integral leafs : after drying at room temperature, the leaves of *L. ericoides* were submmited to an extractive process in ethanol under constant shaking for 2 hours. The extractive solution was concentrated under reduced pressure and the residue obtained was redissolved in ethanol in proportional quantity of 4 parts of solvent to 1 part of extract.

• Tincture of powdered leaves: After a second drying, the powdered leaves of *L. ericoides* without its superficial constituents were submitted to percolation as reported in the Brazilian Pharmacopoeia (2^{a} edition) and the extractor solvent was a 70% (w/w) hidroethanolic mixture. Starting from the fluid extract previously obtained, the tincture was prepared by dissolution in 20% (v/v) of ethanol.

Analytical methods of the solutions

• Determination of dry residue: A small quantity of both extractive solutions were evaporated followed by heating in an oven (105ºC) until constant weight. The results were expressed in percentage.

• High Performance Liquid Chromatography: The obtained solutions were evaporated and dissolved in methanol and then submitted to a clean-up procedure in C-18 column. The soluble fraction was injected in the liquid chromatograph in a isocratic system specific to identify sesquiterpene lactones.

• IR spectra: Samples were analysed in KBr pellets and the spectra were obtained using polystirene as reference (1601 cm^{-1}).

Results

The results were expressed in the average of 3 determinations: drying loss: 6.46%; total ash: 3.11%; ethanol and ethyl ether extractable fractions: 20.70 and 5.60 % respectively; extratives content: 14.22%; foam index: 100 and swelling index: 4.10 (ethanol and water) / 5.70 (ethanol). The dry residues of the extractive solution and tincture were 1.20% and 2.16% respectively.

The analysis in HPLC indicated the presence of sesquiterpene lactones predominately in the extractive solution 1. Also data from IR spectra showed carbonyl bands around at 1750 cm^{-1} indicating the presence of sesquiterpene lactones. The results of the phytochemical profile showed the presence of the following compound classes: antocianidins, antocianins, flavonoids**,** tannins, saponins, terpenes, steroids, and phenolic compounds.

Conclusion

• The analysis of the phytochemical profile indicated the presence of the same secondary metabolites isolated and / or identified in the circadianes studies previously reported in the literature; The pharmacognostic analytical parameters determined for *L. ericoides* show a informative character about this species; The determination of extractable fraction enabled the selection of a suitable solvent that can be used in analytical methods for the investigation of chemical constituents; Ethanol extractive solution 1 showed the same chemical profile as the methylene chloride extract that have been earlier performed by other researchers.

Acknowledgements

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AM-26 OPTIMIZATION OF THE ANALYTICAL CONDITIONS TO EVALUATE THE SKIN PENETRATION OF KETOCONAZOLE BY TAPE-STRIPPING

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Introduction

The skin antifungal therapy with imidazole derivatives, such as ketoconazole, can be conducted by oral, parenteral and topical routes.

The efficacy of topical route requires an adequate drug uptake by the skin, during a period of time, to cause the pharmacological activity.

The therapeutic response will depend on the following processes: (a) drug delivery from its semisolid dosage form, (b) penetration of the drug into the skin layers, (c) elimination rate of the drug by the skin, and (d) production of the desired response.

Some authors have been adopting the nomenclature *dermatopharmacokinetics* or *bioavailability of topical formulations* for the study of the first three processes [1]. Therefore, evaluating the delivery and skin penetration of the drug from its semisolid dosage form becomes important.

Dermatophyte infections occur mainly in the stratum corneum. Therefore, the determination of ketoconazole at this site permits a direct measure of the drug concentration in its target site [2]. The determination of the profile of drug concentration in the *stratum corneum versus* time can be used to demonstrate the bioequivalence of topical products [3].

A methodology employed with this aim is the tape-stripping, which involves dosage form application to volunteer's ventral forearm, *stratum corneum* removal with adhesive-tapes after defined application times, with subsequent extraction and drug analysis employing a previously validated method [3].

Objectives

The aim of this work was to establish optimum extraction conditions of ketoconazole from adhesive tapes (Transpore®), which will be employed in the tape-stripping methodology, to evaluate the extent of drug penetration in the *stratum corneum* from cream formulations.

Experimental Methods

- **Materials**

Ketoconazole (Spectrum, Rio de Janeiro, Brazil); adhesive tape Transpore® (3M, São Paulo, Brazil); HPLC-grade acetonitrile and methanol (Tedia, Ohio, USA); K_2HPO_4 (Grupo Química, Rio de Janeiro, Brazil); isocratic HPLC (Waters® 510 pump and Waters® 486 tunable absorbance detector).

- **Establishment of the quantitative method by HPLC**

Ketoconazole analysis by HPLC was initially established by the evaluation of two different mobile phases: (1) CH_3CN-K_2HPO_4 0,01M (65:35) pH = 6.0 and (2) CH_3CN-K_2HPO_4 0,01M (60:40) pH = 6.8.

The other parameters were kept invariable (RP C_{18} column, injection volume of 20 µl, flow rate of 0.7 ml/min and UV detection at 242 nm).

After choosing the mobile phase, the linearity of the method was investigated. Solutions of ketoconazole were prepared over the concentration range 0.05 to 10 µg/ml, and the correlation coefficient between these concentrations and chromatographic response was calculated. The tailing factor was evaluated and the limit of quantitation was also established.

- **Optimization of ketoconazole extraction from the strips**

The optimization of ketoconazole extraction from the Transpore® adhesive-tapes (1.56 cm^2), which will be employed in the tape-stripping methodology, was studied employing 5 different solvent systems, with or without heating, varying heating time and temperature. The strips were "spiked" with 40 µl of a 50 µg/ml ethanolic solution followed by a rest period for solvent evaporation. Our goal was to establish the solvent system and the extraction condition that provides a good recovery of ketoconazole from the strips.

- **Validation of the extraction methodology**

After establishing the optimum condition for extraction, i.e. the best preliminary recovery of ketoconazole from the strips, a validation of ketoconazole quantitation from them was performed. The accuracy (3 levels of ketoconazole mass in the strips) and precision (n=5 for each level) were evaluated for the responses obtained with the chosen method.

Results and discussion

The mobile phase selected for ketoconazole analysis was CH_3CN-K_2HPO_4 0.01M (60:40) pH=6.8 , because it furnished a good resolution between ketoconazole and the components from the adhesive tape strip. It provided a retention time for the drug of 4.5 min. The method was linear over the concentration range 0.05 to 10 μg/ml of ketoconazole ($r > 0.9999$). The limit of quantitation for ketocoanzole was 0.025 μg/ml or 0.5 ng on the column.

In the study of ketoconazole extraction from the strips, the drug solubility properties and the miscibility of the solvents with the mobile phase were taken in account. Ketoconazole is soluble 1 in 2 of $CHCl_3$ and 1 in 9 of MeOH; partially soluble 1 in 100 of CH_3CN, 1 in 72 of THF and 1 in 54 of EtOH; insoluble in H_2O (the solubility characteristics were determined according to USP 24). The chosen solvent system was CH_3CN-MeOH-H_2O (40:40:20) and 10 min heating time in a 50^oC bath. This condition provided recoveries from 84 to 90% of ketoconazole from the strips. Initially, single and binary systems were tested, as well as extraction condition was performed at room temperature, but unsatisfactory recoveries were obtained. For example acetonitrile-water (70:30) and methanol, resulted in recovery range from 50 to 65 %.

Table 1 presents the validation data for the ketoconazole extraction procedure from the strips. Concomitantly, spiked solutions were prepared and submitted to the same heating conditions to access the stability of the drug. No degradation products were found.

Table 1: Validation of the ketoconazole extraction

Levels of ketoconazole mass added to the strips	Mean recovery of ketoconazole from the strips (n = 5)	Standard deviation	Relative standard deviation
2 μg	84.95 %	1.64 %	1.94 %
5 μg	90.30 %	2.52 %	2.80 %
10 μg	87.45 %	2.14 %	2.45 %

Conclusions

The chosen method for ketoconazole extraction from the strips is considered adequate, since it presents good recovery and satisfactory precision data. The chromatographic conditions for ketoconazole analysis were also satisfactory, since a good resolution between the drug and some interferent substances were obtained. It also permits to quantify low quantities of ketoconazole. This will be useful to evaluate differences among drug formulations regarding their topical bioavailability.
In vivo studies, employing tape-stripping methodology to evaluate ketoconazole bioavailability in *stratum corneum* are being planned in our laboratory. We expect these studies to contribute for the best understanding of uptake and elimination kinetics of this drug in the skin.

Acknowledgements

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AM-27 EVALUATION OF SUN PROTECTION FACTOR (SPF) OF PHYSICAL SUNSCREENS BY DIFFUSE TRANSMITTANCE

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Introduction

The increase in UV radiation on Earth due to the ozone depletion and the excessive exposure to sunlight can cause short-term effects as erythema as well as long-term effects such as photoaging and skin cancer. Not only UVB (290-320 nm) but also UVA (320-400 nm) can be responsible. As a result there is a need for broad-spectrum sunscreens with adequate UVA protection [1].

Sunscreens are classified as chemical or physical depending on whether they absorb, reflect and scatter specific wavelenght bands of the radiation. Because physical sunscreens are not wavelenght selective, they are recognized as effective ingredients in broad-spectrum sunscreen products [2]. Titanium dioxide (TiO_2) is the most important physical UV sunscreen currently used because it provides a high level of protection against UVB radiation and also significant UVA protection. Products combining the high UVB protection of TiO_2 with the high UVA protection of zinc oxide (ZnO) have been also commercialized [3].

The photoprotective efficacy of a particular sunscreen is generally expressed as its Sun Protection Factor (SPF). The SPF is a ratio of the time required for a given radiation to produce minimal perceptible erythema (MED-minimal erythema dose) in sunscreen protected skin to the time required for unprotected skin. Thus, the SPF is predominantly an indication of efficacy against UVB light. The SPF is measured by *in vivo* and *in vitro* techniques. The first method, is a complicated and time-consuming procedure because it involves human being volunteers, not really suitable for broad screening during product development. In adittion, the UVA protection factor of sunscreens is difficult to test *in vivo* because of the relative erythemal insensitivity of normal skin to this waveband. A new *in vitro* technique for measurement of sunscreen protection at any wavelenght has been described by Diffey et al. [4].

Results of sunscreen testing using this technique have shown good agreement with the SPF values claimed by manufacturers, and those of reference formulations defined by American and German standards authorities (FDA and DIN).

In this study we have done the quality control tests, as described at US Pharmacopoeia 24, to obtain the degree of conformity of the raw material with the international regulations. We have also obtained the SPF values of a non-absorbing formulation containing micronized titanium dioxide or zinc oxide at different concentrations, trying to establish the relation between the SPF and the concentration of each physical sunscreen, the maximum concentration allowed by FDA for physical sunscreens is 25% for TiO_2 and ZnO.

Experimental Methods

- **Materials**

Cardre ultrafine titanium dioxide (TiO_2) PP from Cardre Inc. and Z-Cote® HP1(ZnO) from Sunsmart Inc. were used as raw materials for this study.

- **Quality Control tests**

The tests of identification and loss on ignition were performed for TiO_2 and characterization, assay, loss on drying, loss on ignition, alcalinity carbonate and color of the solution for ZnO. Both powders were characterized using a scanning electron microscopy ZEISS DSM 940 A operating at 20 KV

- **Preparation of a non-ionic formulation**

A non-ionic formulation was prepared with 10% chembase, 4% isoctil estearate, 4%glicyne, 0.5% Phenoben and H_2O qsp. The TiO_2, physical sunscreens, was added at various concentrations ranging from 2 to 10%.

- ***InVitro* determination of the Sun Protection Factor (SPF)**

The TiO_2 cream with differents concentrations was applied directly on the Transpore™ tape at a surface density of 2mg/cm^2. The transmittance at 290 nm to 400 nm was obtained from three points on the tape, in the Shimadzu UV-2401PC spectrophotometer with coupled integrating sphere.

Results

- **Quality Control tests:**

	TiO_2		ZnO	
	Result	Specification	Result	Specification
Ident.	Red color	Red color	White precipitate HCl 3N insoluble	White precipitate HCl 3N insoluble
Loss on Ignition	2,72%	<0,5%	2,02%	<1,0%
Alcalinity	------	------	Color absence	Color absence
Carbonate solution color	-----	------	White solution	White solution
Assay	------	------	87,4%	99% - 100,5%

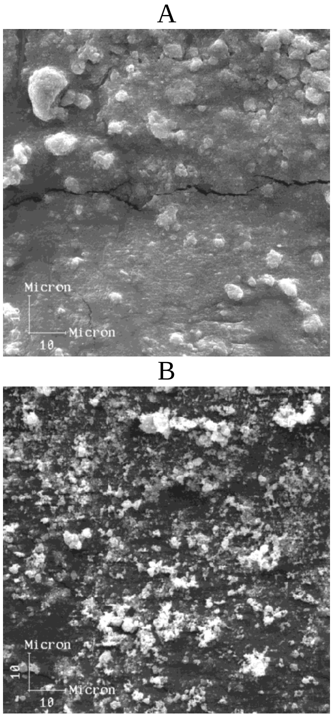

Figure 1: Scanning electron microscopy
A: TiO2 1000x and B: ZnO 1000x

- ***InVitro* determination of the Sun Protection Factor (SPF):**

Concentration TiO_2	SPF
2%	2.71 ± 2.17
4%	-
6%	5.68 ± 0.25
8%	9.40 ± 1.2

Conclusions

Some results from quality control tests showed a certain distance from the specifications, maybe because they were coated and the analysis specifications referred to the uncoated powders.

The preparations showed good stability and pH range at 5.0-6.0.

Regardless of sunscreen protection at any wavelength described by Diffey had been applied in many scientific works, all of them included mixture of chemical and physical sunscreens. Thus far the evaluation of the physical sunscreen (TiO_2) was possible at concentrations 2, 6 and 8%.

Acknowledgement:

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AM-28 A COMPARISON AMONG THREE DIFFERENT METHODS TO DETERMINE THE SUN PROTECTION FACTOR OF SUNSCREENS

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Introduction

The incorporation of sunscreens into daily-wear products and the explosive increase of sunscreen usage by children [1] will inevitable push the sunscreens to their limit. However, as we add more chemical filters at higher levels, the cost for the products increases dramatically along with the potential to cause skin irritation and sensitization. This, at a time when high sun protection factors (SPFs) are being demanded by the consumer, leaves the formulator in a difficult position [2]. Thus the search for substances that might be useful as sunscreens or as excipients that intensify the SPF, and the quality control of sunscreens formulations become more import every day.

The most adequate and traditionally used and officially accepted method in several countries is the *in vivo* method of determination of SPF. The *in vivo* method is expensive and also introduces the ethical consideration of human testing. Moreover, this method is not yet sufficiently practical to be employed routinely in quality control to assure that each batch of sunscreen is adequate for use. It is therefore possible to conclude that the best method to meet these requirements is probably an *in vitro* one. The major advantage of *in vitro* test is that it is a rapid, objective, cost-effective screening methodology.

In previous work [3], several different combinations varying the filters and/or the concentrations used were tested. The formulation that best fits the characteristics desired, that means high SPF with the lowest concentration of filters as possible was the combination of Octyl *p*-Metoxycinnamate at 7.0%, Methylbenzilidene Camphor at 3.0% and Isoamyl *p*-Metoxycinnamate at 3.0%.

Two *in vitro* methodologies for the calculation of the SPFs were compared to an *in vivo* method, that is the unique approved methodology for legal purposes.

Material and Methods

Two different *in vitro* methodologies were used to calculate the SPFs for two distinct formulations, an O/W emulsion and an oil preparation (Formulas 1 and 2)

The first method (method 1) used was described by Mansur *et al.* [4,5]. Each preparation at a final concentration of 0.2μg/mL, diluted in ethanol (emulsion) or isopropanol (oil) were read in triplicate and have their absorbances determined through the use of Shimadzu UV-Visible Spectrophotometer Shimadzu UV-1601.

The second *in vitro* methodology (method 2) used was described by Diffey & Robson [6]. In this method each preparation diluted in ethanol, at a final concentration of 2.0mg/cm^2, were applied on a Transpore Tape® (3M Company) with an area of 7.82cm^2. After solvent evaporation, transmittances have been determined using the Shimadzu UV-2401 PC, together with integrating sphere ISR-2200. Three Transpore Tapes were used for each preparation, where 5 transmittance measures were done (15 measurements per preparation).

The in vivo SPFs were measured as by FDA [7]. The subjects were 20 human volunteers with skin types I, II and III, and the solar ultraviolet stimulator Multiport 601-Solar Light Company was used. Homomenthyl Salicylate at concentration of 8.0% was used as standard.

Results

Formula 1 - Sunscreen oil

Silicone fluid 350 CST		3.0
Octyl stearate		7.5
Octyl *p*-Metoxycinnamate		7.0
Methylbenzilidene Camphor		3.0
Isoamyl *p*-Metoxycinnamate		3.0
Mineral oil	qsp	100

(the amount of each constituent is given in g)

Formula 2 - Sunscreen emulsion

Stearic acid	2.7
Glyceril monostearate	2.0
Mineral oil	2.0
Isopropyl myristate	1.4
PEG-20 cetyl/stearyl ether	2.0
Propylparaben	0.1
Glycerin	3.0
Triethanolamine	0.2
Methylparaben	0.1
Octyl *p*-Metoxycinnamate	7.0
Methylbenzilidene Camphor	3.0
Isoamyl *p*-Metoxycinnamate	3.0
water deionized qsp	100

(the amount of each constituent is given in g)

The data obtained in the analysis of sunscreens formulations by two different methodologies *in vitro* and *in vivo* method are shown in table 1.

Table 1

Values of *in vitro* and in *vivo* SPF of the sunscreens formulations

Sunscreens formulations	SPF *in vitro* method 1	SPF *in vitro* method 2	SPF *in vivo*
Oil (Formula 1)	20 ± 0.47	11.8 ± 0.7	8.7 ± 2.1
Emulsion (Formula 2)	22 ± 0.05	12.3 ± 1.2	14.4 ± 1.2

Statistical Analysis was carried out using STATISTICA – statsoft, version 6.2, based on Wilcoxon Matched Paired Tests. The results showed that Methods 1 and 2 are statistically different with p values of 0.02771 and 0.04310 for formula 1 and formula 2, respectively. Method 1 and “in vivo” Method are also statistically different with p values of 0.02771 and 0.04312 for formula 1 and formula 2, respectively. Method 2 when compared to “in vivo” Method showed no statistically significant differences between them with p values of 0.11586 and 0.0796 for formula 1 and formula 2, respectively.

Some works in the literature describe the method of Mansur (method 1) as convenient and reliable for the *in vitro* calculation of SPFs [8,9]. Although being true for preparations with low to medium concentrations of chemical filters, the same does not occur when formulations have high concentrations of filters. Also when a formulation contains physical filters it becomes impossible to analyze it using Mansur’s methodology once the physical filter is insoluble in the solvent used.

Conclusion

The results demonstrated that Diffey & Robson’s methodology provided the best correlation between *in vivo* and *in vitro* methods using the preparation proposed. This in vitro method is much faster and less expensive than using human volunteers, and is a reliable guide to product performance.

Although *in vitro* tests could not substitute the *in vivo* ones for legal purposes, it can provide a formulation tool to identify new filters, optimize combinations of old ones and pre-screen protective formulas prior to *in vivo* testing in humans.

Acknowledgements

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AM-29 ADAPTATION AND OPTIMIZATION OF THE METHOD OF SOD ACTIVITY DETERMINATION FOR USE IN A GEL-CREAM FORMULATION

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Introduction

Superoxide dismutase (SOD) is involved in a complex antioxidant defense system of the skin to the attack of reactive oxygen species. The skin is always in contact with oxygen and now is increasingly exposed to ultraviolet (UV) irradiation that could induce biological responses such as erythema, pigmentation, carcinogenesis, aging, mutagenicity, cell lethality and melanoma induction, and most of these pathologies seem to be mediated by oxygen radicals. This fact has led to the possible therapeutic implications of protective systems. Particularly, SOD has been extensively studied following administration by various routes, including topical therapy. However, the addition of SOD in a formulation requests a special care with the stability of the enzyme because of its macromolecule nature, its possible interactions with others compounds of the formulation and the peculiar instabilities of proteins. Hence, the purpose of this work was the adaptation and the optimization of the activity determination of SOD according to McCord and Fridovich (1969) for the study of the activity stability of the enzyme in a formulation.

Materials and Methods

- **Formulations**

The formulations used in the assays were prepared with carbomer 940, methylparaben, propylparaben, imidazolidinyl urea, cetearyl alcohol and ceteareth 20, glycerin, trietanolamine and distilled water. One of the formulations was added with 0.1% of superoxide dismutase (Dismutin® – BT, Phentafarm).

- **Superoxide dismutase activity determination**

The enzyme activity was determined according to McCord and Fridovich (1969), using xanthine-xanthine oxidase system as superoxide anion source. The best amount of sample and the rate of dilution of the formulation were researched and then changed in the different tests. One unity of superoxide dismutase was defined as the amount of enzyme that inhibits the cytochrome c reduction in 50% under the assay conditions. The blank was obtained from two different ways: as recommended by McCord and Fridovich (1969) and by the addition of the formulation without SOD instead the buffer.

- **Preparation of samples**

The formulations with or without enzyme were diluted in 50 mM phosphate buffer, pH 7.8, contend 0.1 mM EDTA (phosphate buffer), in different rates, according to the test. These suspensions were mixed at different times and velocities for determination of the best conditions.

- **Determination of influence of the formulation in the xanthine-xanthine oxidase reaction**

Different amounts of the formulation without SOD diluted 1:10 with phosphate buffer were added in the blank of the SOD activity determination and the rate of change in the absorbance in 550 nm by a specific time interval was determined.

Results

SOD was diluted 1:10000 with phosphate buffer and different amounts of this solution were used in the determination of the SOD activity, and the best amount to assay was 100 µL as it is showed in the figure 1. Then the formulation without SOD was diluted in the phosphate buffer and the influence of the formulation in the reaction of xanthine-xanthine oxidase (source of superoxide anion) was studied and showed in figure 2.

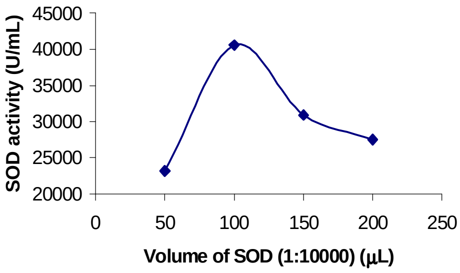

Figure 1. Determination of the best amount of SOD solution in the activity determination.

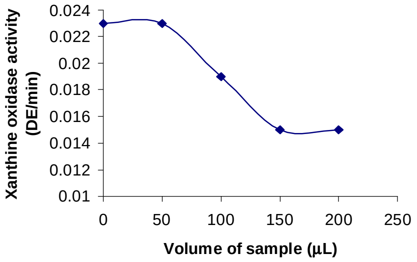

Figure 2. Influence of the formulation in the xanthine-xanthine oxidase reaction.

As it is possible to observe in figure 2, 50 µL of the diluted formulation was the quantity that less interfered in the xanthine-xanthine oxidase reaction. However, 50 µL of the SOD solution was not the best amount to determine the activity in the formulation as it is showed in figure 1. Then, the formulation was diluted 1:5 in phosphate buffer and its activity was compared with the formulation diluted 1:10 in the same buffer. The dilution 1:5 provided the best activity determination (figure 3).

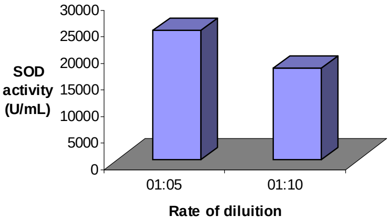

Figure 3. Activity determination for formulations diluted 1:5 and 1:10 in phosphate buffer.

The best time and the velocity of agitation were determined and established as 1 hour and 30 minutes at 500 rpm (figure 4).

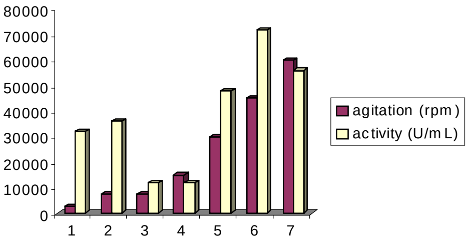

Figure 4. Determination of the best conditions of time and velocity of agitation for sample preparation. 1, 1 min at 2500 rpm; 2, 3 min at 2500 rpm; 3 to 7, 500 rpm for 15, 30, 60, 90 and 120 minutes, respectively.

Conclusion

The method of SOD activity determination could be used for determination of SOD present in a gel-cream formulation under the follow conditions: the formulation must be diluted 1:5 in 50 mM phosphate buffer, pH 7.8, with 0.1 mM EDTA; the suspension of the formulation must be mixed at 500 rpm by 1 hour and 30 minutes; 50 µL of this suspension must be used in the determination of the activity.

Acknowledgements

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AM-30 DETERMINATION OF OXICONAZOLE NITRATE LOTION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC

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Introduction

Oxiconazole nitrate (OxN) (Fig.1) is an antifungal agent of the imidazoles class that has been predominant in fungal therapy . This drug is indicated for topical treatment of cutaneous fungal infections. Results of *in vitro* and *in vivo* studies have indicated that OxN has a broad spectrum of activity against infections caused by dermatophytes, yeast like fungi, molds, and mixed infections due to fungi and gram-positive bacteria [1,2]. These broad – spectrum agent block the synthesis of ergosterol, an essential component of the fungal cell membrane, by binding to cytochrome P-450 [3]. Chemically it is *Z* [(2,4-dichloro-2-imidazol-1-yl) acetophenone]- O –(2,4-dichlorobenzyl)-oxime nitrate and is not yet official in any pharmacopoeia. Previously, the drug has been determined in plasma by highly sensitive gas-liquid chromatographic method [4]. However, an HPLC method to determine OxN in dosage forms has not been reported . Because of their selectivity, sensitivity and overall versatility, the development of reliable and validated HPLC methods has received considerable attention in the quality control of drugs. Therefore, a simple, rapid and reproducible reverse-phase HPLC method for quantification of OxN in raw materials and lotion is presented here.

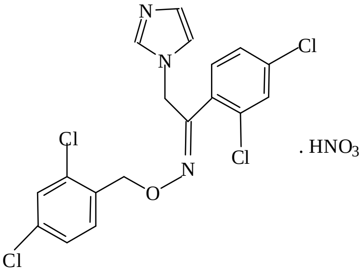

Figure 1- Chemical structure of oxiconazole nitrate.

Experimental

- **Chemical and reagents**

Reference OxN (assigned purity 100.4%) was kindly supplied by Roche Laboratories, while lotion was obtained commercially. Oxiconazole lotion were claimed to contain 10mg (as base) of the drug. Methanol was HPLC grade (Merck, Tedia). Water was glass-distilled.

- **Apparatus and chromatographic conditions**

The HPLC apparatus consisted of a Shimadzu LC-10 A system equipped with a model LC-10AS pump, an SPD-10A variable–wavelength detector (set at 254 nm) and a rheodyne injection valve with a 20 µl loop (Shimadzu, Kyoto, Japan). Chromatograms were recorded and analyzed using a Class- VP® (Shimadzu) chromatographic data system. A Merck RP 8 (125 mm x 4 mm i.d 5 µm particle size, 100 A pore diameter) was used with methanol–0.02M ammonium acetate buffer (85:15 v/v) isocratic as the mobile phase, at a flow rate of 1ml min^{-1}. The HPLC system was operated at ambient temperature (25 ±1°C).

- **Calibrations curves**

A stock solution of 400µg ml^{-1} OxN was prepared in a volumetric flask by dissolving 22.93 mg of OxN (equivalent to 20 mg of oxiconazole) in 50 ml of methanol. Appropriate amounts of the stock solutions were diluted with phase mobile yielding concentrations of 40.0, 60.0, 80.0, 100.0, 120.0 and 140.0 µg ml^{-1}. Triplicate injections of each were made.

- **Procedure for sample preparation**

A quantity of the lotion containing 50.0 mg of OxN was transferred to a 50 ml volumetric flask and methanol added to make up to volume. Five mililitros of this solution was transferred to a 50 ml volumetric flask and mobile phase added to make up to volume, to give a final concentration of 100 µg $ml.^{-1}$.

- **Calculation**

The drug contents of the lotion was determined by referring either to the calibration curve or by sample/equivalent reference substance direct matching .

- **Method validation**

The method was validated by determination of the following operational characteristics: linearity, range, precision and accuracy [5].

Results and discussion

Drug analysis is undertaken during various phases of pharmaceutical development such as formulation and stability studies, quality control and pharmacological testing in animals and man. All these investigations require reliable and validated analytical methods in order to measure drugs in pharmaceutical formulations and biofluids [6]. There have been little or no reported methods for OxN dosage forms. However, the aim of this study was to develop a simple and isocratic HPLC assay for the analysis of this drug in raw materials and pharmaceutical preparation. The choice of the method depends on factors such as the nature of the drug, the complexity of the sample and the intended use. In this study the conditions were influenced by the physico-chemical properties of OxN such as solubility, polarity, UV absorption and interferents. Many commercial pharmaceutical contain benzyl alcohol as a bacteriostatic preservative which can potentially interference with analytical methodologies [7]. During the development of a HPLC method we observed an interfering peak in the analytical procedure that was identified as benzyl alcohol The optimum mobile phase was composed of methanol-0.02M ammonium acetate buffer (85:15 v/v), the flow rate to 1.0ml min^{-1} and detection was monitored at 254 nm. With this mobile phase, the good resolution between the peak of OxN and the peak of benzyl alcohol was obtained. We used the external standard method, because is simple, fast and accurate for sample preparation. The retention time for OxN was about 2.66 min. It results were reproducible with the R.S.D. ranging from 0.49 to 1.63% for between-day studies.

The calibration curves for OxN were constructed by plotting concentration versus peak area and showed good linearity in the 40 - 140µg ml^{-1} range. In the range the percent R.S.D. on the basis of peak area ratios for three replicate injections were found to be between 0.15 - 0.95 %. The inter-day precision was evaluated by comparing the linear regressions of the three standard plots prepared on three different days, over one month period. The representative linear equation for OxN was: $y = 18979.71 + 14893.51x$ ($n = 3$, $r = 0.9999$, $r^2 = 0.9999$). The repeatability of the precision method was studied by assaying six samples of lotion, at same concentration, during the same day under the same experimental conditions. The results obtained through the HPLC analysis with lotion shown 102.0% of OxN and R.S.D. of 0.16%. The results of recovery test of OxN lotion are in agreement with spiked amount of reference substance.

Conclusion

The HPLC method developed in this study has the advantage of simplicity, precision, accuracy and convenience. Moreover, the method uses simple reagents, with minimum sample preparation procedures, encouraging its application in routine analysis.

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AM-31 METHODS FOR THE DETERMINATION OF ETIDOCAINE IN DENTAL CARTRIDGES: A COMPARATIVE STUDY.

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Introduction

The pain control is a fundamental part of the successful practice of dentistry. The dental clinical practice, actually, requires three local anesthetic agents: a short, an intermediate and a long-duration of action [1]. Selecting the most appropriate local anesthetic for the treatment of the patient is possible to attend differently at each appointment. Etidocaine Hydrochloride (EH) is a local anesthetic of the amide type, which is designed as butanamide, N-(2,6-dimethylphenyl)-2-(ethylpropylamine)-monohydrochloride, an analogous of lidocaine. It differs from lidocaine by the addition of a propyl in place of an ethyl group at the amine end, and an addition of an ethyl group on the α carbon in the intermediate chain. Etidocaine hydrochloride has a rapid onset similar to lidocaine, but has a longer duration of action [2,3]. Many studies showed the efficacy of etidocaine in reducing postoperative pain following dental procedures, and some have suggested a post anesthetic analgesia and decreased incidence of severe pain in the postoperative period when etidocaine is used rather than an intermediate duration local anesthetic agent [4]. No analytical procedures are reported in pharmacopoeias for determination of EH in pharmaceutical formulations. A review showed that there are very few works about its physical and chemical assays. The methods published for the quantitative determination of EH are gas chromatography [5,6,7] and high-performance liquid chromatography [8,9,10] using biological fluids. We developed analytical methods to use in routine determination of EH in the injectable pharmaceutical preparation studied [11,12,13]. This work presents the results obtained and a comparative study among the developed methods.

Experimental

- **Materials**

Etidocaine hydrochloride (EH) reference (assigned purity 99.99%) and pharmaceuticals containing etidocaine injectable 1.5% (Duranest®) were generously supplied by Astra-USA. All other chemicals were analytical grade.

- **Methods**

The methods developed were: visible spectrophotometry by ion-pair with bromocresol green, nonaqueous titration, first, second and third derivative UV spectrophotometry and HPLC. The extractive colorimetric method is based on the formation of the yellow-green ion-pair complex extractable in chloroform at pH 4.6 and that exhibits a maximum absorbance at 625 nm. The derivative UV spectrophotometric methods were performed using 0.1N hydrochloric acid as diluting. The maximum absorbances were presented at 277.5, 268.8 and 270.2 nm to first, second and third derivative respectively. The HPLC system Shimadzu SLC-10A (Kyoto, Japan) consisted of LC-10AD pumps, using an isocratic system, sample injector with a 20 µL sample loop, SPD-10A model UV-VIS detector at 254 nm and C-R6A Chromatopac integrator. Chromatographic experiments were performed on a µBondapack™ CN RP (3.9 x 300 mm) 10 µm and 125 A silica column (Waters USA). The mobile phase consisted of acetic acid, methanol, acetonitrile and high purity water (40:10:20:930). The flow rate used was 0.5 mL min. The methods were validated according to their respective operational characteristics [14] as range, linearity, accuracy, precision (repeatability and intermediate precision) and specificity. The data obtained from the determination of EH in dental cartridges by the different methods developed were evaluated by ANOVA and TUKEY.

Results and discussion

The results obtained in the validation methods are shown in Table 1**.**

Table 1 – Statistical data* for calibration curves

Statistical Parameters	1	2	3	4	5
Range (μg mL^{-1})	2.0-6.0	80.0-480.0			50.0-200.0
b	0.1563	0.0003	0.0003	0.0013	934.330
a	0.0567	0.0012	0.0015	0.0032	-250.149
R.S.D.(%)	1.89-2.95	0.0-1.3	0.39-5.8	0.0-0.52	0.66-1.94
r	0.9986	0.9998	0.9999	0.9999	0.9999

* Average of three determinations.

1-Visible spectrophotometric (VS), 2-First UV Derivative Spectrophotometry (DS), 3- Second UV DS, 4- Third UV DS, 5- High performance liquid chromatography (HPLC), b: angular coefficient (slope), a: intercept, R.S.D.: relative standard deviation, r: Pearson's correlation coefficient

The results obtained by the applicability evaluation of the methods are presented in Table 2.

Table 2 – Statistical evaluation of the experimental data obtained from etidocaine hydrochloride determinations by different methods.

Statistical Parameters	1	2	3	4	5	6
Mean*	14.68	15.02	15.30	14.71	15.23	14.88
S^2	0.314	0.298	0.143	0.141	0.075	0.073
R.S.D.(%)	2.13	1.98	0.93	0.96	0.49	0.49
N	5	6	8	8	8	8

1-VS, **2**- Nonaqueous titration (NT), **3**-First UVDS, **4**- Second UVDS, **5**- Third UVDS, **6**-HPLC, *EH mg mL^{-1} injectable solution

The results obtained from the ANOVA test showed there was significant difference among the developed methods. Thus, the Tukey test was performed to find these differences.

Conclusion

The developed methods showed acceptable linearity, precision and accuracy and are suitable for routine analysis of EH in the injectable pharmaceutical preparation studied. Through the Tukey test it was possible to find equivalence among the following methods: Second UVDS-VS; HPLC-Second UVDS; NT-HPLC; First UVDS-Third UVDS.

Acknowledgements

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AM-32 BARBALOIN IN COMPLIMENTARY PHARMACEUTICALS AND DIETARY SUPPLEMENTS

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Introduction

Aloe is a perennial plant; it belongs to the Liliaceas family and xerofitas groups. Originally from the northeast of Africa, it has widely spread through the Mediterranean, Caribbean and America [1,2].

The dried latex of this plant is widely used for manufacturing food products, pharmaceuticals and cosmetics because of its aromatic properties, bitter taste, cathartic activity of anthraquinones and other pharmacological activities such as emolliency, epidermic repairer, anti-inflammatory, antiasmthatic, analgesic, antipyretic and hypoglycemic properties.

The barbaloin or aloin, a mixture of two diastereoisomers, aloin A and aloin B, is an anthrone C-glycoside component of aloe; the others main components are aloesin, homonataloin, aloinoside A and B, aloenin A and B, and aloe-emodin [2,3].

The analytical techniques most frequently used to determine barbaloin are gravimetric, spectrofotometric and chromatographic, including liquid chromatography in reverse phase. High Performance Liquid Chromatography (HPLC) identifies barbaloin from a variety of products containing aloe [3].

In Chile, complimentary pharmaceuticals are commercialized under the regulation of "Decreto Supremo N° 855/98" [4]. Since legislation has not yet incorporated the concept of dietary supplement. These products need to have an analytical quality control.

This work quantifies the amount of barbaloin in complimentary pharmaceuticals and dietary supplements commercialized in the Region of Valparaíso, Chile using HPLC.

Objective

Determination of barbaloin by HPLC in complimentary pharmaceuticals and dietary supplements with aloe.

Methodology

Barbaloin content was determined using an HPLC equipped with quaternary pump system (Lachrom L-7100). A Merck C18e LiChroCart® column (5um x 250 x 4.6 mm, Merck, Darmstadt, Germany) was used as analytical column at ambient temperature. Elution program was a linear gradient acetonitrile- water HPLC grade, from 100% to 80% water in 30 min, followed by a second linear gradient from 80% to 100% water in 45 min, at a flow rate of 1 mL/min. Wavelength UV- Visible detector (Lachrom 7400) was set at 360nm. All solution to be analyzed were injected at the volume of 50 µL.

Standard solutions of barbaloin (*Aloe barbadensis* Miller, Sigma), 97% purity were kept in the dark at -18°C under nitrogen.

The complimentary pharmaceuticals and dietary supplements with aloe were bought in the market place. To analyze solids (soft and hard capsules) and semisolids (gel) 1 g of sample was weighed accurately. 0.5 g was weighed to analyzed tablets and 2 mL were measured for solutions.

With the exception of all products, except drops, were extracted with methanol, filtered using PTF 0.2 mm filters and injected directly for analysis.

Results and Discussion

Retention time of barbaloin was 11 min. A linear correlation was found between the concentration of barbaloin (range 0.5 – 300 µg/mL) and its chromatographic answer, r 0.99 and r^2 0.98.

The system and method were exact and coefficient of variation (CV) 1,36 and 0,57. The calculated CV was within the USP acceptance range (CV ≤ 2). The recuperation percentage was 100.42 %. Repeatability and reproducibility parameters presented a CV 3.35 and 1.34.

The limit of detection (L.D.) was 24 ng/mL and the limit of quantitation 74 ng/mL.

Barbaloin is unstable in solution, within the five first days a slight reduction of its concentration can be observed, 0.63 % of degradation (Figure 1).

A faster degradation is observed reaching a 90% of loss between the 15 to 20 days. Thus, preparation and analysis of the samples must be carry out in less than smaller five days.

Table 1 shows that the alkaline hydrolysis causes an important degradation of barbaloin, nevertheless, picks of degradation products are not shown at this anthrone retention time.

Due to barbaloin chemical structure it is possible that rupture of the ring heterocyclic near ketonic group is originated by an oxidating atmosphere. These degradation compounds do not modify the chromatographic signal of barbaloin.

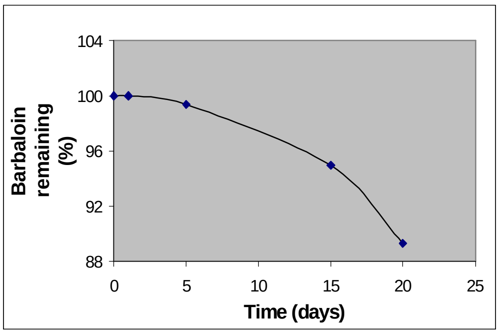

Figure 1: Degradation kinetics of barbaloin solutions stored to -18°C.

Acid hydrolysis will produce the destruction of the glycoside link in position 10. The reaction does not influence the measurement of anthrone.

Photolysis causes an important degradation of barbaloin but its degradation products do not alter the chromatographic signal.

In summary, determination of barbaloin must be carried out avoiding sunlight, extreme pH conditions and oxygen.

Table 1
Not altered barbaloin after each process.

Process	% Barbaloin
Standard	100
Oxidation	52.3
Acid Hydrolysis	52.7
Alkaline hydrolysis	1.53
Photolysis	82.17

The amount of barbaloin in dietary supplements and pharmaceuticals complimentary commercialized in Region of Valparaíso is variable (Table 2). Most of the product dosage was under the colagoge dose (4 and 12 mg) [2]. One of the products showed, by unitary dosage, an amount of barbaloin with action colagoge or digestive tonic, without laxative effect. Barbaloin in the gels of aloe was not detect, then the gastrointestinal action, may be due an effect of its high soluble dietary fiber content. Barbaloin was not detected in only one of the dietary supplements.

Table 2
Barbaloin by unitary dosage in market products.

Product	Barbaloin (μg)	Pharmaceutical Dosage	Classification
1	7.9	Capsule	D.S.
2	13.7	Capsule	D.S.
3	≤ L.D.	Capsule	D.S.
4	532.5	Capsule	P.P.
5	303.5	Capsule	P.P.
6	55.4	Capsule	P.P.
7	7124.2	Tablet	P.P.
8	1213.0	Tablet	P.P.
9	16.6	Gel	D.S.
10	≤ L.D.	Soft capsule	D.S.
11	≤ L.D.	Solution	P.P.

P.P. : Pharmaceutical product
D.S. : Dietary supplement
L.D. : Limit of detection

Conclusion

The validated HPLC method, implemented to quantify barbaloin, allows its determination in pharmaceuticals complementary and dietary supplements.

The results showed that all the products commercialized in the Region of Valparaíso, Chile contained an amount less than 20mg of barbaloin. This dose has a digestive tonic effect in human.

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BE-01 COMPARATIVE BIOAVAILABILITY BETWEEN TWO TABLET PROPRANOLOL FORMULATIONS IN HUMAN HEALTHY VOLUNTEERS

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Introduction

To determine the bioavailability of two propranolol 40mg tablet test formulation from Fundacão para o Remédio Popular (FURP), Brazil, compared to a propranolol reference formulation (Propranolol Ayest 40 – Wyeth) in 23 healthy volunteers, who received a single oral dose (80mg) for each propranolol formulation.

Experimental methods

- **Subjects**.

Twenty three healthy male volunteers ranging in age from 18 to 37 years (mean ± SD: 24.04 ± 5.01), in body weight from 55 to 95 Kg (mean ± SD: 66.83 ± 9.31) participated in the study (Table I). All subjects had a normal medical history and revealed no abnormalities on physical examination, routine laboratory tests, and electrocardiogram. Written informed consent was obtained from each subject and the clinical protocol was approved by the Human Ethics Committee of the Federal University of Pernambuco State.

- **Protocol**

The subject were no smoker and were instructed not to drink caffeine- or alcohol-containing beverages for at least 12 hours before and during the study day. The study was performed following open, randomized, tree-period crossover design with one-Week washout period between each treatment. During each period, the subjects were hospitalized at 10 pm having already eaten a normal evening meal. After a 10-hour fast they received a single 80mg oral dose of Propranolol 40mg FURP, Propranolol 40mg FAR-MANGUINHOS, Propranolol Ayerst 40mg - Wyeth tablets with 200ml tap water. They were fasted for 2 hours after drug administration and they received a standardized breakfast.

Results and discussion

- **Plasma Propranolol Levels.**

An indwelling venous catheter was then introduced and kept patent with a diluted heparin solution. Venous blood samples for propranolol determination were taken up to 24h after drug administration. Samples were centrifuged at 4000 rpm at room temperature for five minutes, plasma was then removed and frozen until time of assay. In this paper will be presented data from Propranolol 40mg from FURP and Propranolol 40mg Ayest from Wyeth.

Table 1. Demographic and anthropometric data of healthy male volunteers

	Age (years)	Weight (Kg)	Height (m)	CMI
V1	20	56	1.70	19.38
V2	26	58	1.73	19.38
V3	31	73	1.68	25.86
V4	21	63	1.69	22.06
V5	23	67	1.88	18.96
V6	18	63	1.77	20.11
V7	20	62	1.67	22.23
V8	26	63	1.69	22.06
V9	37	67	1.67	24.02
V10	30	63	1.67	22.59
V11	18	70	1.74	23.12
V12	18	55	1.65	20.20
V13	20	79	1.80	24.38
V14	27	70	1.80	21.60
V15	20	74	1.75	24.16
V16	21	95	1.89	26.60
V17	27	75	1.75	24.49
V18	24	57	1.60	22.27
V19	20	57	1.68	20.20
V20	30	61	1.61	23.53
V21	29	72	1.68	25.51
V22	25	61	1.60	23.83
V23	22	76	1.78	23.99

The plasma propranolol assay was based on the procedure developed by Roger *et al*. Briefly, an internal standard atenolol was added to the patients' samples and the standard samples prepared by spiking control plasma with stock solutions of propranolol. Analyses were performed on a Waters liquid chromatograph (HPLC) equipped with a Waters 474 fluorescence detector with an excitation wavelength of 230nm and emission filter of 340nm.

Waters micro-Bondapack C18 reversed phase column. The mobile phase monosodium phosphate pH 3,0/acetonitrile/ methanol (50/35/15).

The mean propranolol plasma concentrations vs. time-curves obtained after a single oral administration of each propranolol tablet formulation are show in Figure 1.

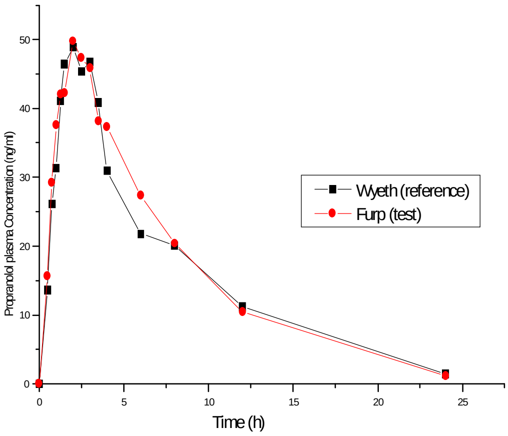

Figure 1 - Mean plasma propranolol concentration versus time curves after single dose administration of two propranolol 40mg tablets of the test and reference formulations.

The areas under the propranolol plasma concentration vs. Time curves from 0-24h were calculated by applying the linear trapezoid rule. The others pharmacokinetic parameters obtained included the $AUC_{0-\infty}$, the maximum plasma concentration (C_{max}), and the time taken to achieve this concentration (T_{max}), the elimination rate constant (Ke) and the terminal half-life ($t_{1/2}$.). The table II shows the geometric mean and 90% CI values for all parameters. AUC_{0-24} and C_{max} data were analyzed statistically by using a parametric test (ANOVA for log-transformed data). The software used included Linear/logarithmic trapezoidal rule, version 2001, Pharmkit version 2.10, Bioequivalence Program for Two-Period Crossover Studies, version 6.21. Microsoft Excel version 7. Table III summarizes the bioequivalence analysis of individual AUC_{0-24} and C_{max} for the propranolol formulations.

Conclusion

Based on the results and considering the ANVISA-MS requirements, we found that Propranolol FURP is bioequivalent to propranolol Ayest – Wyeth for both, the rate and the extent of absorption after single dose administration.

Table 2 – Mean pharmacokinetic parameters for 23 volunteers after the administration of propranolol formulations.

Parameter	Propranolol FURP	 WYETH
AUC_{0-24} ($ng.h.ml^{-1}$)		
Geom. mean	339.0	306.5
90% CI	292.0-384.2	265.3-354.0
$AUC_{0-\infty}$ ($ng.h.ml^{-1}$)		
Geom. mean	461.4	454.4
90% CI	411.8-516.8	399.5-516.7
C_{max}		
Geom. mean	63.5	65.6
90% CI	56.9–70.8	58.1–74.0
Ke (h^{-1})		
Geom. mean	0.13	0.12
90% CI	0.11–0.15	0.10 – 0.14
$T_{1/2}$ (h)		
Geom. mean	5.5	5.9
90% CI	4.5–6.5	4.9–7.1
T_{max} (h)		
Median	2	2
Range	0.75 – 4	1 – 3.5

Table 3 – Statistical analysis of individual $AUC_{(0-24)}$, $AUC_{(0-\infty)}$, C_{max} and ratios for the propranolol formulations

	Statistical analysis Geom. mean	 90% CI
AUC_{0-24} % ratio	109.63 %	98.7 – 121.7
C_{max} % ratio	95.91 %	86.2 – 106.8

Acknowledgements

This work was supported by a grant from the Agência Nacional de Vigilância Sanitária (ANVISA) – Ministry of Healthy, Brazil.

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BE-02 CEPHALEXIN BIOEQUIVALENCE STUDY IN HUMAN HEALTHY VOLUNTEERS

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Introduction

Cephalexin is a semi-synthetic analogue of cephalosporin C and is active against *Streptococcus pneumoniae*, *Neisseria gonorrheae* as well as against *Staphylococci* and *Strep. viridans*. Cephalexin is also indicated in the treatment of respiratory tract infections, otitis media, skin and soft tissue infections, bone and joint infections, genitourinary and dental infections.

Cephalexin is available from several manufacturers for oral administration in suspension, tablets and capsules containing 250 or 500 mg of cephalexin.

Absorption of cephalexin from the gastrointestinal tract is almost complete, usually 80-100% of an orally administered dose. Peak serum concentrations attained at 1h are approximately 15-20 and 25-30 mg/L after a 500 mg and a 1 g dose respectively.

Objective

The aim of the study was to evaluate, in human volunteers, the performance of a Cephalexin oral suspension formulation (Cephalexin, oral suspension – 250mg/5mL, by Eurofarma laboratórios Ltda., Brazil) against one standard Cephalexin oral suspension formulation (Keflex®, oral suspension – 250mg/5mL, by Eli Lilly do Brasil Ltda., Brazil).

Experimental methods

The study consisted of an open study of 24 (twenty-four) healthy volunteers of both sexes (12 male and 12 female), with age between 18 and 50 years and weight between 55 and 95Kg with ± 15% of the acceptable range of weights for men according to height and frame.

The volunteers were enrolled into the study only if they were found to be fit and well as assessed by clinical and laboratory test evaluations performed within eighth weeks prior the study commencement. The volunteers realized electrocardiogram (ECG), haematology tests (hemoglobin, hematocrit, differential count of band neutrophils, segmented neutrophils, eosinophils, basophils, lymphocytes and monocytes), platel count, biochemistry tests (urea, creatinine, total bilirrubin, total protein, albumin, fasting blood glucose, alkaline phosphatase, SGOT, SGTP, total cholesterol, triglyceride, uric acid, gamma GT) urinalyses in the pre-study period and post-study period. The Sorology tests for hepatitis B, C, HIV; Beta-HCG (for women) and faeces tests were realized only in the pre-study period.

Each dosing comprised of 250mg (5mL) of Cephalexin oral suspension formulation or 250mg (5mL) of Keflex® oral suspension formulation, allocated to the appropriate dose randomization code. The Cephalexin formulations were taken with 200mL of mineral water.

Subjects were required to attend the Clinical Pharmacology Unit at approximately 21:00h on the evening prior to each dose. The subjects remained in the Unit until 12h after dosing.

All volunteers arrived at the Clinical Pharmacology Unit of the Federal University of Ceara having eaten a normal evening meal. All volunteers were required to remain fasten until two hours after dose administration in the following morning, when a xanthine-free standard breakfast were available. A xanthine-free standard lunch was provided after five, eight and twelve hours after dose. No other food was permitted during the “in-house” period.

Liquid refreshment was permitted ad *libitum* after lunch, but xanthine containing drinks (including tea, coffee, cola) were avoided.
Smoking was not allowed during the “in-house” period.

Concomitant medication was to be avoided where possible during the study. Any medication, including over the counter medication, was to be recorded.

Alcohol consumption was limited throughout the study period and avoided completely during 48 hours prior to each dose.

Blood sampling for plasma drug assay were taken from a suitable forearm vein pre-dose and at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 2.50, 3.00, 4.00, 6.00, 8.00, 10.00 and 12.00 post-dosing. On each occasion, one 8mL sample was taken either via ‘butterfly’ or syringe into clean tube containing anti coagulant. The actual times of sampling were recorded using 24 hours clock notation.

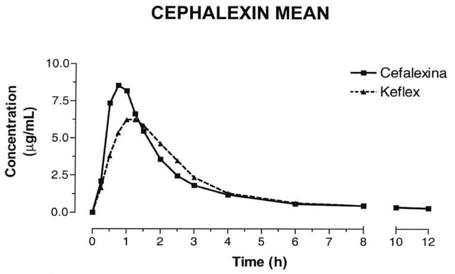
Cephalexin plasma concentrations were determined by a validated method using high-pressure liquid chromatography coupled to a mass spectrometer (LC-MS-MS).

Results and discussions

Volunteers demonstrated good tolerance for both products. Just a volunteer demonstrated adverse reactions during the clinic study. It was a middle intensity headache for 2 hours, which needed 1 tablet of Paracetamol, 500mg.

The maximum concentration reached (Cmax), the Areas Under the Curve from the time of dosing to the time of the last observation (AUC $_{(0\text{-}12h)}$) were compared **.**

Cephalexin 250mg/5mL oral suspension formulation Cmax geometric mean ratio was 126.8% (90% CI = 112.8-142.5) of Keflex® 250mg/5mL oral suspension formulation.
Cephalexin 250mg/5mL oral suspension formulation AUC$_{(0\text{-}12h)}$ geometric mean ratio was 103.9% (90% CI = 97.1-111.1) of Keflex® 250mg/5mL oral suspension formulation.



CEPHALEXIN MEAN

Figure 1. Cephalexin and Keflex Concentration profiles.

The graphic indicates mean Cephalexin, concentration data vs time. It demonstrated that both formulations had similarities bends for identical time periods.

Conclusion

Since the 90% confidence interval for C_{max} ratio was inside the 70-143% interval proposed by the *European Community* and since 90% confidence interval for AUC$_{(0\text{-}12h)}$ ratio was inside the 80-125% interval proposed by the Food and *Drug Administration* and the *European Community*, it can be concluded that Cephalexin 250mg/5mL oral suspension formulation, from Eurofarma Laboratórios Ltda. is bioequivalent to Keflex® 250mg/5mL oral suspension formulation, from Eli Lilly do Brazil Ltda. for the extent of absorption.

Acknowledgements

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BE-03 BIOEQUIVALENCE EVALUATION OF ORAL SUSPENSION CONTAINING CEFADROXIL

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Introduction

Cefadroxil is a first generation semi-synthetic cephalosporin administered orally. It is active against aerobic gram-positive bacteria but has limited activity against gram negative bacteria. Cefadroxil has been mainly used to treat infections in urinary tract, skin and some infections in the respiratory tract.

Cefadroxil is quickly absorbed by the GI tract after oral administration. The rate of absorption and peak serum concentrations are not affected when the drug is administered with food. Peak serum cefadroxil concentrations are attained within 1 – 2 hours after oral administration in healthy adults. Average 10 – 18 µg/mL are observed in serum when 500 mg of cefadroxil is administered [3].

Cefadroxil is administered orally and in Brazil it is manufactured by five companies. Laboratories have been interested in developing generic products containing cefadroxil. However, there have been no studies on the bioequivalence of such products.

According to the FDA – USA Bioequivalence Division and the official Brazilian guidelines - RDC 10/01 [1,2] the bioequivalence evaluation must be performed by *in vivo* comparisons of drug absorption speeds and amounts for test and reference formulas. For such evaluation, accessible amounts of biological fluids, such as blood or urine, are analyzed concerning drug and metabolic concentrations.

Objectives

The objectives of this work were to develop and validate an analytical and adequate method to quantify cefadroxil in human plasma of healthy volunteers through high performance liquid chromatographic (HPLC) in order to evaluate the bioequivalence of two products, in oral suspension form, manufactured by two different companies and commercialized as similar products.

Materials and Method

A group of 24 volunteers, 12 males and 12 females, participated in the bioequivalence study. All of them were informed about the characteristic of the study which had been fully approved by the ethics committee related.

Values initially obtained for the parameters C_{max} and AUC_T were utilized to verify the effects of product, group and period through variation analysis (ANOVA). The two products are considered bioequivalent if C.I. 90% for the relation between C_{max} and AUC_T of both, calculated at log transformation basis, are between 80 and 125% [1,8]. The procedure of the bioequivalence study was a randomized, cross over and open, forming two groups, A and B. The medication was administered in a single dose of 500 mg. At phase I, group A volunteers had the reference product, while group B had the test product. At phase II, performed after one week (wash out period), the procedure was repeated and the groups were inverted. Plasma samples obtained at adequate times in order to determine cefadroxil plasmatic concentration were frozen at -20° C until drug quantification. The developed and validated analytic methodology to quantify cefadroxil in plasma through high performance liquid chromatography (HPLC), using acetaminophen as internal standard, has proved to be simple and suitable.

Validation parameters such as specificity, precision and accuracy intra and inter-days, linearity, recovery, limit of quantification and stability were determined in order to assure the result confidence. Plasma samples extraction containing cefadroxil was made through the precipitation of proteins with acetonitrile [4,6]. Conditions for HPLC were: Luna Phenomenex® column (150mm, 4.60 mm ID, 5µm), mobile phase was constituted by 0.02M monobasic sodium phosphate and methanol at a proportion of 85:15 (v/v), and final pH was adjusted to 3.5 with acetic acid; the flow and column temperature utilized were 1.4 mL/minute and 23°C respectively. UV detection was performed at 230 nm.

Cefadroxil quantifying in volunteer plasma samples who had reference and test products was performed in parallel to the calibration curve at six different concentrations and quality control plasma samples that have been analyzed in double and at three different concentrations within the range that was intended to be determined.

Results and Discussion

As validation results, the developed method was proved to be specific for cefadroxil and acetaminophen (internal standard), showing good separation of plasma components with retention time of 3.1 and 4.3 minutes respectively. The specificity was evaluated through the analysis of blank , hemolized and lipemic plasma.Precision was between 6.7 and 12.1% for samples analyzed in the same day (intra-day) and between 10.8 and 14.1% for samples analyzed in different days (inter-days) and accuracy ranged between 90.2 and 101.3% and 95.9 and 102.7 % for intra-day and inter-day analysis respectively. The mean extraction recovery was 73.6 % and the limit of quantification (LOQ) was 1.5 µg/mL.

Calibration curve, blank and quality control plasma samples remained stable for one month at a temperature of –20°C. Drug stability during three freezing and defrosting cycles was also evaluated and adequate results were obtained. The resulting organic extracts remained stable for 24 hours at ambient temperature, after reconstitution with mobile phase.

There was no significant statistical difference for pharmacokinetic parameters (Table 1). The mean “plasmatic concentration *vs* time” curves presented differences within accepted limits according to standards established by FDA and ANVISA - National Agency of Sanitary Vigilance, through RDC 10/01 (Figure 1). The variation analysis revealed the presence of product effect in C_{max} and AUC_T parameters.

The confidence intervals were 105.27 to 114.51% and 108.55 to 114.43% for C_{max} and $AUC_{T,}$ respectively.

The difference found for test and reference products in C_{max} and AUC_T parameters is within acceptable confidence limits of 80 to 125% according to ANVISA and FDA and both products are considered therapeutically equivalent.

Table 1. Mean results of pharmacokinetic parameters after administration of 500 mg of cefadroxil to 24 healthy volunteers.

	Reference			**Test**		
	t_{max} (h)	**C_{max} (ug/mL)**	**AUC_T (ug*h/mL)**	**t_{max} (h)**	**C_{max} (ug/mL)**	**AUC_T (ug*h/mL)**
Mean	1,27	19,10	57,63	1,25	21,06	64,93
C.V.(%)	28,37	24,79	18,28	31,21	26,79	24,52

The bioequivalence evaluation of the products was based on the absorbed amount - AUC_T: area under the “plasmatic concentration versus time” curve and absorption speed, C_{max}: maximum plasmatic drug concentration after oral administration, and t_{max}: necessary time for reaching C_{max} [5]. Statistic result analysis was performed according to FDA–USA recommendations and United States Pharmacopeia – 24th edition [2,7].

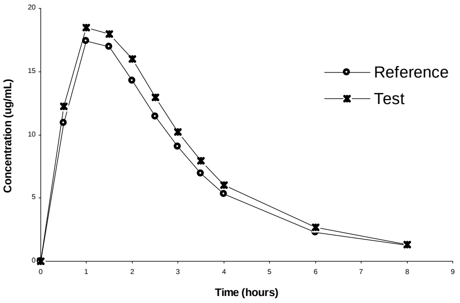

Figure 1. Mean serum cefadroxil concentration *vs* time after administration of 500 mg of cefadroxil to 24 healthy volunteers.

Conclusion

Thus, according to results presented and after adding data from literature concerning C.I. (80 – 125%), that show the importance of this parameter as the major indication of bioequivalence [8], it was concluded that the test and reference products are bioequivalent.

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BE-04 BIOEQUIVALENCE EVALUATION OF FORMULATIONS CONTAINING HYDROCHLOROTHIAZIDE.

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Introduction

Hydrochlorothiazide is a thiazide diuretic administered orally. This drug acts directly on the reabsorption mechanism of electrolytes in the nephron distal tubule resulting in improved excretion of sodium, chloride and water. Natriuresis can be followed by loss of potassium and other electrolytes such as magnesium, phosphate, calcium, bromide and iodide [8]. After oral administration, hydrochlorothiazide is quickly absorbed. Diuretic action starts within two hours after administration and its peak occurs within three to six hours. The presence of food in the GI tract enhances absorption. The linkage to plasmatic proteins is 68% and the drug is extensively distributed throughout the organism linked to eritrocytes. Distribution volume is 3.6 to 7.8 L/kg [8].

Aiming at the introduction of generic medication in Brazil, it is interesting to evaluate bioequivalence of formulations containing hydrochlorothiazide [4, 5].

Bioequivalence will be determined through comparison of maximum plasmatic drug concentration after oral administration (C_{max}), necessary time for reaching C_{max} (t_{max}) and area under the “plasmatic concentration versus time” curve (AUC_T) [5, 9, 11]. For that reason, it is necessary to develop a method to quantify hydrochlorothiazide in biological fluids [1, 2, 3, 6, 7, 10].

Objectives

The objectives of this work were to develop and validate an analytical method to quantify hydrochlorothiazide in human plasma through high performance liquid chromatographic (HPLC) and UV detection and to evaluate bioequivalence of hydrochlorothiazide tablets from two different manufacturers that are sold as similar medications. One of them was named reference (R, Drenol®) and the other was named test (T, hydrochlorothiazide FAR-MANGUINHOS).

Material and Methods

- *Casuistic*

In a randomized, cross-over and open study, the medication was administered in a single dose of 50 mg to 25 healthy volunteers, 14 females and 11 males (mean aged of 24.7, mean height of 169.7 cm and mean weight of 64.7 kg).

Hydrochlorothiazide tablets were administered in the morning after an eight-hour fasting and were taken with 300 mL of water. Volunteers had after four hours after administration.

- *Sample Collection*

Blood samples were collected at 0; 0,5; 1; 1.5; 2; 2.5; 3; 3.5; 4; 6; 8;12; 24; 36; 48 and 60 hours after drug administration. Samples were centrifuged and plasma was frozen at – 20°C until drug quantification.

- *Sample Preparation*

Plasma samples from volunteers were analysed in parallel to calibration curve, blank and quality control samples.

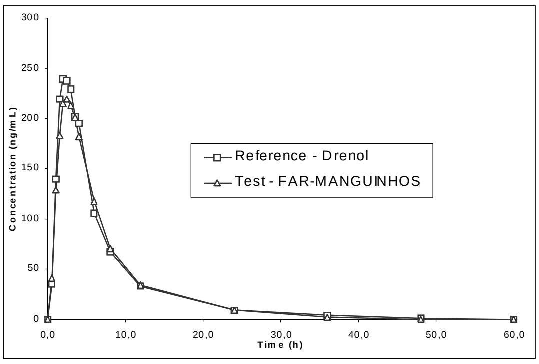
Hydroflumethiazide was utilized as internal standard (IS), being both drug and IS extracted from plasma using tert.-buthyl ether in the plasma. To each tube were added 1.0 mL of plasma 25 µl of a 10 µg/mL IS solution in methanol and 5mL of tert.-butyl ether mixed by vortex for 2 minutes. Plasma was freezed and the organic phase was filtered in a HV Millex unit with a Durapore® membrane (13 mm of diameter and 0.45 µm of pore). The filtered material was transferred to a conic tube and evaporated. The residue was reconstituted in 1000 µL of mobile phase to be quantified by HPLC.

- *Chromatographic Conditions*

For separation, a Lichrocart Merck® column (55mmx3mm ID, 4µm) was used. The mobile phase was constituted by a mixture of water, tetrahydrofuran and acetonitrile at a proportion of 85:10:5 (v/v). The flow and column temperature utilized were 1.0 mL/min and 40°C respectively. UV detection performed at 272 nm.

- *HPLC Method Validation*

The validation was performed according to official brazilian guidelines [5] determining specificity, recovery, calibration curve, quantification limit, precision, accuracy and stability.



	Drenol®			FAR-MANGUINHOS		
	t_{max} h	C_{max} ng/mL	AUC_T ng*h/mL	t_{max} h	C_{max} ng/mL	AUC_T ng*h/mL
Mean	2,3	281,3	1748,0	2,6	244,2	1676,9
C.V.%	43,44	38,80	33,16	26,64	24,73	22,43

Results and Discussion

The developed method has proved to be specific for hydrochlorothiazide and internal standard, showing good separation of plasma components with retention time of 1.9 and 3.4 minutes respectively. The mean extraction recovery was 81.9%. A linear relation between concentrations between 10 and 500 ng/mL was obtained. The limit of quantification (LOQ) was 10 ng/mL. Precision varied between 3.0 and 4.7%. Accuracy varied between 96 and 113%. Calibration curve, blank and quality control samples remained stable for 180 days at a temperature of – 20°C for two freezing and defrosting cycles.

The resulting organic extracts remained stable for 24 hours at ambient temperature, after reconstitution with mobile phase.

The mean “plasmatic concentration versus time” curves for test and reference products were similar (Figure 1).

Figure 1. Mean serum hydrochlorothiazide concentration *vs* time after administration of tablets containing 50 mg of hydrochlorothiazide to 25 healthy volunteers.

There was no significant statistical difference for pharmacokinetic parameters (Table I).

Table I. Mean results of pharmacokinetic parameters after administration of tablets contents 50 mg of hydrochlorothiazide to 25 healthy volunteers.

The multivaried analysis (ANOVA) performed indicated the absence of product, group and period effects. The confidence intervals were 83.9 to 97% for C_{max} and 92.2 to 106.6% for AUC_T .

Conclusion

According to the presented results, the developed and validated analytical method to quantify hydrochlorothiazide in plasma has proved to be adequate for bioequivalence study, presenting specificity, recovery, calibration curve, LOQ, precision, accuracy and stability [5].

The test and reference products are bioequivalent according to ANVISA, FDA and EMEA criteria [9, 13].

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BE-05 BIOEQUIVALENCE OF NIMESULIDE TABLETS OF BRAZILIAN MARKET

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The nimesulide (4-nitro-2-phenoxy-methanosulphonanilide) is a non acidic nonsteroidal anti-inflammatory drug (NSAID). It is a sparingly water-soluble drug and exerts its pharmacological activity through various mechanisms of action [2]. In Brazil there are several oral pharmaceutical forms marketed, with nimesulida in a dosage of 100 and 200 mg. Therefore studies to establish the biopharmaceutical quality of some batches of this products became interesting and useful to evaluate the actual situation.

The present study was designed to perform *in vitro* (dissolution kinetics) and *in vivo* (bioequivalence) biopharmaceutical evaluation of two commercial products available in Brazil: The reference product (R) and the test product (T). Initially a method was development and standardized for evaluation of dissolution kinetics of nimesulide tablets.

Dissolution kinetics for samples from three batches of R an three of T were analyzed through k_s (dissolution rate constant) and $t_{50\%}$ (time for dissolution of 50% of the drug in the dosage form), obtained from dissolution profiles. In the development of methods to analyze the dissolution kinetics of nimesulide in tablets, several different condition medium was tested [1,9]. Results of preliminary study indicate that IV condition, which use paddle method at 50 rpm, medium water and sodium lauryl sulfate (SLS) and the IXV condition, which use paddle method at 50 rpm and buffer phosphate medium (pH 8.0), show that the release rate increased. The XIV condition showed minimum variation in the results in the tablets analyzes in the same assay and close the physiologic medium.

Evaluation of the concentration curve “percentage nimesulide dissolved versus time” demonstrates that the products A e B showed close profiles and highest rate release. On the other hand, the product D demonstrates to be deficient, whereas the product C showed an intermediate profile.

The parameter K, $t_{50\%}$ and DE clearly demonstrates above, that indicates the best results to the products A and B. The half-life of dissolution ($t_{50\%}$) is useful to provide the time necessary to dissolve 50% of drug. Therefore, the products witch the rate release of drug is fast, the value of $t_{50\%}$ is relatively lower (2.49 and 4.45 minutes) adequate to formulation of immediate-release. The product D the values was elevated, 18.73 minutes for one batch to dissolved 50% of drug and the product C the values was intermediate but less then A and B. This affirmation was the same to dissolution efficiency (DE).

A study of kinetic of dissolution process using the zero order and first order models were carrying out for all products. By the linearity of dissolution curve, was taking the respective correlation coefficient (r). Observed that the models more adequate (highest r) were the first order for the all products, it was expected to solids formulation of immediate-release.

Results showed k_s values of 0.1417 min^{-1}; 0.1506 min^{-1} and 0.1138 min^{-1} (R) and 0.2540 min^{-1}, 0.1965 min^{-1} and 0.1557 min^{-1} (T), and $t_{50\%}$ values between 4.6 and 6.09 min (R) and 2.73 and 4.45 min (T).

The products assayed in this study may be considered pharmaceutical equivalents because it was having the drug with the same chemical nature, same molar dose and same formulation. In this case, the products are marked in Brazil like similar interchange will expect these products will be equivalents in clinical efficacy e safety. The *in vivo* study designs are employed to conclusion definitive about bioavailability and bioequivalence of products in study and later evaluation of correlation between parameters of dissolution and absorption.

Bioequivalence assay was crossover and randomized. Blood samples were collected throughout a twelve hours period of administration of R and T to twenty-four fasting volunteers [5,7,8].

The developed and validated analytic methodology to quantify nimesulida in plasma through high performance liquid chromatography (HPLC), using Phenacetin as internal standard, has proved to be sensitive and reproducible. Ultraviolet detection was carried out at 230 nm. Validation parameter such as specificity, precision, intra and inter-day accuracy, linearity, recovery, quantification limits and stability were determined in order to assure the results confidence [3,4,6].

Plasma samples extraction containing nimesulida was made through liquid-liquid extraction with dichloromethane. Conditions for HPLC were: Supelcosil LC-18 column (150 mm, 4.6 mm ID, 5 µm), mobile phase was constituted by phosphate buffer 4.4 pH and methanol at proportion of 60:40 (v/v), and final pH was adjusted to 6.8; the flow 1.0 mL/minute.

Nimesulide quantifying in volunteer plasma sample who had reference and test products was performed in parallel to the calibration curve at five different concentrations and qualify control plasma sample the have been analyzed in double and at three different concentration within the range that was intended to be determined

Bioequivalence was assessed through pharmacokinetic parameters C_{max} (peak plasma concentration), t_{max} (time to reach C_{max}) and AUC_T (area under the plasma concentration vs time curve) for R and T. Results were submitted to statistical analysis according to the ANVISA and FDA-USA and 90% confidence intervals (90% C.I.) were calculated for R and T C_{max} ratios and T and R AUC_T ratios. Average C_{max} and AUC_T values for R and T were, respectively: 9.22 µg/mL and 9.41 µg/mL; 58.98 µgxh/mL and 58.52 µgxh/mL. 90% C.I. for C_{max} and AUC_T were respectively, 96.73 - 101.44 % and 99.42 - 105.31%.

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BE-06 CONTRIBUTION OF PHARMACEUTICAL TECHNICAL COMPLAINT TO PHARMACOVIGILANCE

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Introduction

The problems related to the quality of medicaments in Brazilian market have been target of the frequent accusation. In public institutions, this get worse with a kind of purchase procedure known as “bidding” which benefits the minor price of products without being accompanied on good quality one. Recent efforts in reinforcing and implementing regulation have not yet changed this profile.

Otherwise, even in developed countries, problems with quality of pharmaceuticals, sum up with medication errors and adverse reactions to put on risk patient health. This is originating a more integrative approach of post-marketing surveillance of medical technologies, including medicines, as Med Watch, for example.

In this context, since 1996, the Pharmacovigilance Center of Ceará (CEFACE), a research and extension center of Group for the Prevention of Improper Use of Pharmaceuticals (GPUIM) has collected and received adverse drug reaction reports (RAMs) and technical complaints (QT) about pharmaceutical products.

Technical complaints are defined, in this way, as some evidence of deviation of the quality standards required to product register, as well as some suspect of therapeutic inefficiency.

The objective of the present work was to show pharmaceutical technical complaint reports collected and assessed by CEFACE in order to evaluate the potential contribution of this strategy to pharmacovigilance .

Methods

The team of CEFACE does weekly monitoring in four hospitals of Fortaleza-Ceará (Hospital Universitario Walter Cantidio, Hospital de Pneumologia e Cardiologia de Messejana, Maternidade Escola Assis Chateaubriand, Hospital Distrital Nossa Senhora da Conceição. In these places, the reporting of RAM and QT suspected are collected (spontaneous reports) and investigated (active search).

In particular, this work is related to analysis of QT reports collected by CEFACE from 1996 (November) to 2000 (November).

The QT cases were registered using a yellow card of CEFACE as described by Coelho et al., 1999. This card has fields to put information about RAM and QT reports. In the QT cases, some parameters such as number of medicament lot, manufacture date, manufacturer and the description about the problem were required.

The CEFACE’s procedure about QT cases varied in according to type of complaint. Initially, the problem was clarified in order to know if the QT was clearly related to the product or to its manipulation and/or administration. In cases of definite technical problem, relating to pharmaceutical quality, the complain was sent to Sanitary Vigilance of Ceará. In others cases, an investigation about storage, manipulation, prescription and administration procedures was done in order to value if they could cause that problem.

Before decisions, the conclusions were discussed in meeting of CEFACE’s team which is formed by pharmacologists, pharmacists, Pharmacy and Medicine students. If necessary, an assessment of pharmaceutical technology professional could be included.

According to the result, a pharmaceutical intervention could be done through an orientation about correct procedures in order to prevent the ocurrence of similar events.

Each report was coded and entered into a computerized database of CEFACE. The data processing was done in Epi-Info program.

Results and discussion

In the study period, the CEFACE registered 65 cases of QT in the involved hospitals. Among the direct reports, nurses submitted 43 (74%) and pharmacists 13 (20%) cases. Physician reporters submitted only 9 cases (6%) (Table 1). The nurses are considered important reporters in QT cases because they are responsible, in general, for preparing and administration of drugs and so they are near to potential problems related to QT.

About the types of QT reports, colour changes of the products were the most frequent (57%) following therapeutic inefficiency (22%). Other types were shown in Table 2. Colour changes occurred, in the most of cases, after dilution of substances in big volumes. After the general evaluation, several reports were categorized as storage, manipulation, prescription and administration errors.

The annual distribution of QT reports revealed a bigger number of cases in 1999. This increasing reports of QT occurred at the same time of Brazilian media's boom of denounces of falsification of medicaments (Table 3). It is interesting to note the concentration of therapeutic inefficiency suspect increased also in this period. The majority of these suspicions was related to local anesthetic drugs.

In addition, it is good to say, that the results of cases sent to State Sanitary Vigilance are unknown by CEFACE.

Conclusion

In our work, some types of hospital technical complaints were shown. The medicaments produced without the suitable guarantee of quality, problems related to manipulation, prescription and administration of drugs were observed and evaluated by CEFACE.

We wanted to show that QT monitoring could be a good advice for Pharmacovigilance development, performing a complementary role in assuring a safe use of medicines. By this way, is necessary that this service be able to explain the observed problems using laboratory tests.

It should be emphasized that surveillance can be done on hospitalar physician materials and related products too.

Table 1- Distribution of QT reports by type of reporter from 1996 (November) to 2000 (November)

Reporter	Number of reports	%
Nurses	43	74
Pharmacists	13	20
Physicians	9	6
TOTAL	65	100

Source: GPUIM/CEFACE

Table 2- Number of QT reports by type of product alteration from 1996 (Nov.) to 2000 (November)

The storage. manipulation. prescription and administration problems of medicaments		
Type	Number of reports	%
Colour change	37	57
Therapeutic inefficiency	14	22
Precipitated formation	6	9
Appearance and colour change	3	4.5
Foamy aspect after reconstitution	1	1.5
PARTIAL TOTAL	61	94
Others problems		
Type	Number of reports	%
Presence of strange body	3	4.5
Suffocating sensation *	1	1.5
PARTIAL TOTAL	4	6
TOTAL	**65**	**100**

Source: GPUIM/CEFACE

* Suffocating sensation occurred in patient when the administered product was substituted by one of another mark. The reaction disappeared with the suspension of the actual drug and use of the product with the same mark.

Table 3- Annual distribution of QT reports in CEFACE

Year	Number of reports	%
1997	02	3.4
1998	14	21.5
1999	46	70.5
2000	03	4.6
TOTAL	65	100

Source: GPUIM/CEFACE

Acknowledgements

This work was supported by Brazilian National Research Council (CNPq), and the Federal University of Ceara.

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QC-01 METHODOLOGY VALIDATION AND DISSOLUTION PROFILE OF LAMIVUDINE TABLETS (150 mg)

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Introduction

Lamivudine (also known as 3TC) is a drug belonging to the nucleoside analogue class, being used in the treatment of HIV types 1 and 2 infections[1]. It has been widely used in Brazil for the treatment of AIDS, in association with another drugs, with good results[2].

Aiming to assure the product safety and compliance with the federal government needs of low cost and high quality generic drugs to AIDS public program, it urges to evaluate the quality of the pharmaceutical preparations available.

Among the pharmacopoeial tests applicable to tablets, the dissolution studies and profiles are important parameters to assess the pharmaceutical preparation performance. They are useful in future *in vitro/in vivo* correlation studies.

There is no lamivudine monograph in Brazil's current accepted pharmacopoeias[3]. Therefore, the validation of analytical methods, especially dissolution test itself and profile is important to the Ministry of Health in the acquisition processes.

Objectives

- To study different dissolution test parameters for lamivudine tablets
- To validate the dissolution test for lamivudine tablets
- To compare the tablets dissolution profiles from batch to batch of same laboratory and of different laboratories
- To assess the quality of lamivudine tablets produced in Brazil

Experimental

Material

- 72RL Hanson dissolution tester
- UV 160A Shimadzu UV-Visible spectrophotometer

Standard

Lamivudine reference standard (batch AWS535A 99.7% w/w), obtained from Glaxo Wellcome

Samples

Lamivudine reference tablets (150 mg) obtained from Glaxo Wellcome (Epivir®) and lamivudine test tablets obtained from laboratories A, B, C and D

Reagents

Hydrochloric acid (Merck); potassium chloride (Vetec) - analytical grade.

Methods

The dissolution methods used for testing the tablets from the five laboratories were compared.

The dissolution medium (distilled water vs. buffer pH 1.2), apparatus (paddle vs. basket) and time (30 vs. 60 minutes) were evaluated. The volume of dissolution medium was 900 mL, the speed was 50 rpm, the wavelength was 270 nm and tolerance was Q = 80%

Final samples concentration: 1.67 mg/100 mL.

The dissolution profile study was made with three batches of test and reference tablets produced by four different laboratories. Distilled water was used as dissolution medium, paddles as stirring element, speed of 50 rpm and sampling times on 2.5; 5; 15; 30; 45 and 60 minutes.

The profiles were compared between batches of the same laboratory and between all the other batches, using the difference factor (f1) and the similarity factor (f2). In order the curves to be considered similar, f1 values should be 0-15 and f2 values should be 50-100 [4].

Results

There is no significantly difference between the results with distilled water and buffer pH 1.2, except for the batches of one of the laboratories tested. It suggests that there might be a problem in these tablets production. The results obtained with paddles and baskets were no significantly different, however the dissolution profile test was selected to be performed with the paddles due to USP 24 specifications [5].

Four of the five batches tested showed approximately 100% release in 30 minutes, with a small release increase when tested in 60 minutes.

The results obtained in the dissolution profile study were: similarity between the reference batches (Figure 1) and the batches produced by A (Figure 2). Among the batches produced by B, only one showed similarity (Figure 3). None of the batches produced by C showed similarity (Figure 4).

The similarity results between all the batches tested is shown in the Table. There is similarity only between the three reference batches.

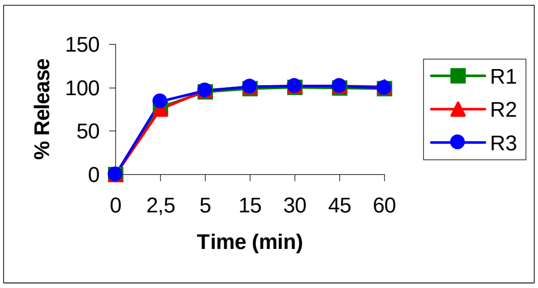

Figure 1 - Dissolution profile of three batches of reference pharmaceutical preparation

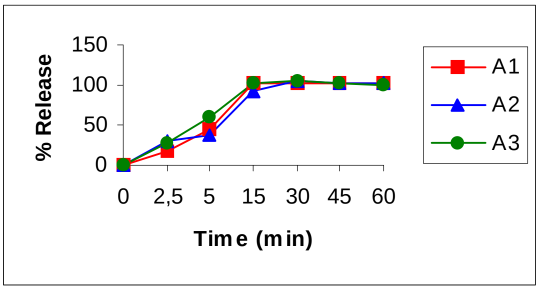

Figure 2 - Dissolution profile of three batches of laboratory A

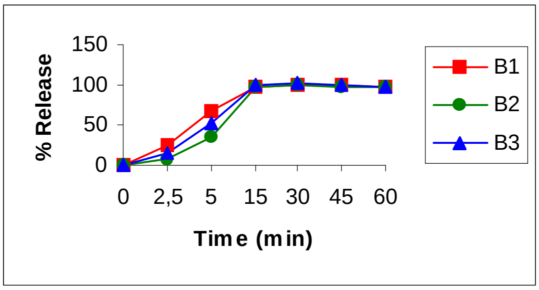

Figure 3 - Dissolution profile of three batches of laboratory B

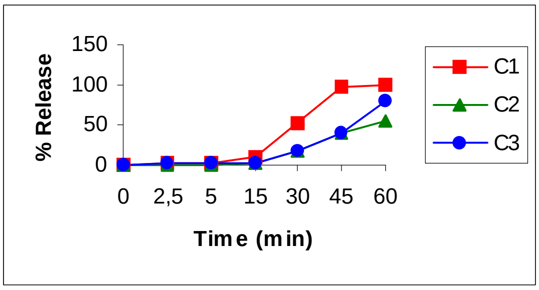

Figure 4 - Dissolution profile of three batches of laboratory C

Table 1- f1 and f2 values for reference and test batches

Batch	f1	f2
R2	1.91	84.06
R3	2.47	74.49
A1	21.42	24.65
A2	21.56	25.39
A3	17.10	29.90
B1	14.55	30.90
B2	23.75	21.20
B3	18.87	25.59
C1	54.03	9.63
C2	79.32	5.46
C3	74.14	6.15

Conclusion

- The dissolution test for lamivudine tablets was validated in the following conditions: distilled water as dissolution medium, paddles as stirring element and 30 minutes as test duration.
- There is little variation in the drug release between 30 and 60 minutes dissolution profiles.
- There is more similarity when comparing batches of the same laboratory other than comparing them with the reference batch.

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QC-02 COMPARISON OF DISSOLUTION PROFILES THROUGH SIMILARITY FACTOR, f2, IN BIOEQUIVALENT CARBAMAZEPINE TABLETS

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Introduction

Dissolution testing is very important in the quality control of solid dosage forms. The common test in the United States Pharmacopeia (USP) [1] for immediate release forms, usually considers a single point test. In order to obtain more and better information, pharmaceutical scientist has currently performed comparisons of dissolution profiles. In the last years Moore and Flanner [2] proposed a model independent method, -the similarity factor f2- to quantitatively compare such profiles.

The Biopharmaceutical Classification System (BCS), intends to classify drugs using their solubility and permeability characteristics [3]. According with some indications of the FDA [4], drugs with high solubility and high permeability could be proposed for a waiver of the bioequivalence study, based only in the dissolution behavior. In vivo performance of Class 2 drug -low solubility high permeability- is very dependent on the characteristics of the formulation, since once dissolved these type of drugs are fast absorbed. In this class of drugs a change on the dissolution profile would be probably reflected in changes on the *in vivo* profile of plasma concentrations.

The goal of this study was to assess if the similarity factor f2, applied to some tablets of a Class 2 drug, carbamazepine, would be a good parameter to estimate the bioequivalence of 2 marketed formulations, using the innovator as the reference.

Materials and Methods

- **Dosage forms**

3 immediate release carbamazepine tablets (innovator, Form A and Form B), commercially available, manufactured by different pharmaceutical companies, were used. All tablets were analised according to the USP 24 monograph of carbamazepine.

- **Dissolution profiles**

USP 24 conditions were used: 900 ml of water containing 1% of sodium lauryl sulfate, apparatus 2, 75 rpm. 12 tablets of each formulation were tested and samples were collected at 1-2-4-6-8-12-20-30-45- and 60 minutes.

- **Similarity factor**

The formulations were considered as similar if f2 was equal or higher than 50.

$f2 = 50\log\{[1 + 1/n\ \Sigma\ (Rt-Tt)^2]^{-0,5} \times 100\}$

n= number of samples

Rt= % dissolved at the time t of the reference product

Tt= % dissolved at the time t of the test product

Bioequivalence study: a 3 way cross over study involving 21 healthy subjects was performed.

Pharmacokinetics parameters

AUC, Cmax, t max, MRT, total, maximum and early exposure were assessed. They were statistically compared according with the recommendations of the FDA.

Results

All the formulations fulfilled the requirements of the USP 24 for carbamazepine tablets. Mean dissolution values are shown in Table 1. The relative standard deviation was lower than 10 % in the first 4 minutes and lower than 5% at the times higher than 6 minutes for all formulations.

Table 1: % dissolved of the formulations

Time (min)	Innovator	Form A	Form B
1	12.81	6.99	12.41
2	27.07	18.04	26.21
4	39.68	33.15	29.65
6	45.39	44.02	38.07
8	49.32	50.87	43.42
12	54.99	59.36	51.98
20	62.69	67.64	63.04
30	70.08	74.06	71.69
45	78.50	80.08	79.5
60	82.38	84.39	88.74

Table 2: pharmacokinetic parameters

Parameter	Innovator	Form A	Form B
AUC (mgh/l)	187.1±53.0	192.5±41.8	191.6±49.8
Cmax (mg/l)	2.31 ± 0.43	2.21± 0.34	2.19± 0.52
Tmax (h)	8	12	8
MRT (h)	68.0 ±19.7	72.0± 19.1	73.5± 19.1

Statistical comparison of the pharrnacokinetic parameters demonstrated that Form A and Form B were bioequivalent with the innovator. When the plasma concentrations were treated in order to calculate the early exposure, the values were also into the bioequivalence confidence intervals.

In order to calculate a reliable similarity factor f2, we selected dissolution times not only lower than 85% dissolved, to maintain the discriminatory power of the test, but also very early times to properly define all the profile. At the first times selected, 1and 2 minutes, we could appreciate some differences on the Form A, but they were probably due to a little difference on the disintegration time of the tablets.

Similarity factor f2 for the Form A was 64.5 and for Form B was 66.1, both compared with the innovator product. These values demonstrate that dissolution profiles are similar to the innovator, and are in agreement with the results obtained in the in vivo study. In the case of carbamazepine, the conditions of the dissolution test are critical. Once dissolved, the drug has a very good permeability through the intestinal mucose. If the test really correlates with the in vivo behavior, then the similarity factor applied to the dissolution profiles would be an interesting tool to make some inferences about the bioequivalence of different formulations.

Acknowledgements

We thank the CEDETEF of the University of Chile for its support.

Conclusions

Comparison of dissolution profiles in carbamazepine tablets through the similarity factor f2 resulted in a good agreement with the results obtained in the *in vivo* study.

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QC-03 *IN VITRO* DISSOLUTION PROFILES OF CARBAMAZEPINE POLYMERIC SOLID DISPERSIONS – COMPARISON OF DATA BY FIT FACTORS AND DISSOLUTION EFFICIENCY

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Introduction

In recent years more emphasis has been dedicated to the design and interpretation of dissolution testing. *In vitro* dissolution testing provides useful information during the drug development process. In our previous work [1] the feasibility of spray-drying solid dispersions from aqueous dispersions of carbamazepine (CBZ) associated to β-cyclodextrin (βCD) and hydroxypropyl methylcellulose (HPMC) was demonstrated. Dissolution tests were performed in order to evaluate the release profiles of carbamazepine from solid dispersions containing different ratio of CBZ:βCD:HPMC, named MA (50:15:35), MB (50:25:25) and MC (50:35:15). The release profiles were dependent on the ratio of βCD:HPMC and the rank order (MC>MB>MA) is in agreement with the amount of polymer employed in view to obtain a sustained release of the drug.

The purpose of this work is to compare the release profiles of CBZ from these solid dispersions by two statistical methods: fit factors and dissolution efficiency.

Experimental methods

- **Materials**

Carbamazepine was purchased from Galena (São Paulo, Brazil). β-cyclodextrin was obtained from Roquette (France) and HPMC (Methocel K100LV®, DOW Chemical Company) was supplied by Blanver (São Paulo, Brazil). The solid dispersions were prepared by spray-drying using a mini spray dryer Büchi 190 (Büchi, Göppingen, Germany).

- ***In vitro* dissolution testing**

Dissolution tests were carried out using a Pharma Test dissolution tester (USP XXII) linked to a Hewlett Packard 8452A spectrophotometer with multicell transport. The paddle speed was set at 100 rpm and an USP simulated gastric fluid without enzymes (1000 mL) was employed, at 37 °C, for 2 hours. In order to obtain *sink* condition, samples of solid dispersions corresponding to 20 mg of drug were placed in gelatin capsules. Samples were automatically collected each 10 minutes, filtered at 0.45 µm and CBZ was assayed by spectrophotometric method at 286 nm. Three batches of each formulation (MA, MB and MC) were tested in gastric fluid and its dissolution profiles were compared to that of CBZ powder.

- **Statistical methods**

Fit factors

The similarity (f_2) and difference (f_1) factors were determined according to the following equations [2;3]:

$$f_2 = 50 \bullet \log\left\{\left[1+\left(\frac{1}{P}\right)\sum_{i=1}^{P}(R-T)^2\right]^{-1/2} \bullet 100\right\}$$

$$f_1 = \left\{\left[\sum_{i=1}^{P}|R-T|\right]\Big/\left[\sum_{i=1}^{P}R\right]\right\} \bullet 100$$

Where *R* and *T* represent the dissolution measurements at *P* time points of the reference and test, respectively. The number of sample points was limited to no more than one, once any of the product reaches 85 %, as recommended by Shah and co-workers [3].

Dissolution efficiency

The dissolution efficiency (D.E.) was calculated based on a model independent method, using the equation described below. It is defined as the area under the dissolution curve up to 120 min, expressed as a percentage of the area of the rectangle described by 100 % dissolution in the same time.

$$DE = \frac{AUC_{(0-120\,\mathrm{min})}}{AUC_{100\%}} \times 100\%$$

Results and discussion

The dissolution profiles of the three spray dried formulations versus CBZ in USP simulated gastric fluid are shown in figure 1. The data are illustrated graphically by plotting the mean dissolution profile for each formulation with error bars extending to standard deviations at each dissolution time point. As the intervals for formulations MA and MB overlap, the mean dissolution profiles may be equivalent to each other. Similarly, after 50 minutes the intervals of CBZ and MC also overlap. Since it may be difficult to conclude that CBZ and MC, as well as MA and MB are significantly different, a mathematical comparison was further performed. For each formulation batch f_1 and f_2 comparisons were made. The three batches of the same formulations were considered equivalent (data not shown), and the percentage of CBZ released from each formulation represents the mean of the 18 dissolution profiles obtained (6 from each batch). The f_1 and f_2 comparisons are illustrated in Table 1.

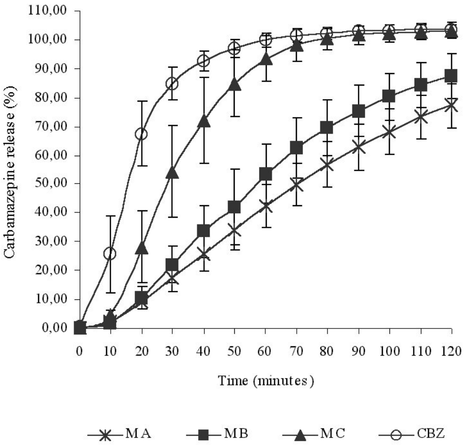

Figure 1. *In vitro* dissolution profile of MA, MB, MC and CBZ in gastric fluid

Table 1. f_1 and f_2 values for each comparison

Comparisons	f_1*	f_2
CBZ x MA	52.1 or 108.6	12.3
CBZ x MB	42.6 or 74.3	13.8
CBZ x MC	27.8 or 38.5	27.0
MA x MB	19.9 or 16.6	**50.9**
MA x MC	81.9 or 45.0	20.6
MB x MC	52 or 34.2	24.4

* The first f_1 value is obtained when the first formulation of the left column is set as reference

As one can observe, in all cases f_1 was higher than 15, which represents significant differences among the formulations. However, the f_2 value of 50.9 (in bold) is very close to the threshold of similarity acceptance ($50<f_2<100$)[3]. Considering these results, the analysis of de D.E. was important in the evaluation of the difference between MA and MB dissolution profiles.

The D.E. data are listed in Table 2. The ANOVA demonstrated statistically significant differences among the formulations (α=0.1). The Tukey test confirmed that all formulations where significantly different from each other. In fact, the D.E. variation of 11.6 % between CBZ and MC and of 26.2 % between MC and MB are in agreement with the f_2 values obtained. The threshold f_2 value of 50.9 for MA *versus* MB could be easier interpreted by a decrease lower than 10 % (8.1 %), nevertheless, a decision based on the statistical test does not support the f_2 result. Based on the f_1 and D.E results, these formulations may be classified as different.

Table 2. D.E. values of CBZ, MA, MB and MC

Product	D.E. (%)	RSD (%)
CBZ	86.0	4.4 $^{(n=6)}$
MA	40.1	13.7 $^{(n=18)}$
MB	48.2	13.6 $^{(n=18)}$
MC	74.4	7.5 $^{(n=18)}$

Conclusion

The statistical methods used to evaluate the differences among the dissolution profiles revealed conclusions which would not be expected when a simple exploratory data analysis is performed. These results give importance to the careful choice of statistical methods used in the interpretation of the dissolution studies. The selection of a model which may describe the curves shapes could be further evaluated since the methods described so far have not clarified the data interpretation.

Acknowledgements

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QC-04 NIMESULIDE TABLETS: CORRELATION BETWEEN BIOAVAILABILITY AND DISSOLUTION DATA

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Introduction

The value of dissolution as a quality control tool for predicting *in vivo* performance of a drug product is significantly enhanced if an *in vivo-in vitro* (*IV-IV*) relationship (correlation or association) is established. Nimesulide (NMS) is a non-steroidal anti-inflammatory drug that has been present in the Brazilian market for the last 10 years, mainly in solid dosage forms. At present moment there is no official monographs for this drug in the known compendia. For drugs with very low water solubility, as NMS, the dissolution test is particularly important to evidence problems in formulation and manufacturing that can result in significant changes in bioavailability. The Biopharmaceutical Classification System – BCS [1] provide an estimate for *IV-IV* correlation probability, arranging the drug substances into four categories according to their solubility and permeability properties. The NMS belongs to the class II of BCS (low solubility, high permeability) which states that an *IV-IV* correlation is probable.

Goals

The objective of this work was to provide elements for validation of a dissolution method for NMS immediate release tablets. So, at first it was investigated different experimental conditions that could change the NMS dissolution rate from tablets for further relationship study with bioavailability data in volunteers.

Methodology

- **Dissolution test**

The dissolution profiles were obtained by the rotating-paddle method (apparatus 2 according USP Pharmacopeia), using a volume of 1000mL of different media and two stirring speeds (50 and 75rpm). The media employed were: aqueous solutions with sodium lauryl sulfate (NaLS) (0.5; 1.0; 1.5 and 2.0 % w/v), aqueous solutions with polysorbate 80 (0.5; 1.0; 1.5 and 2.0 % w/v) and phosphate buffer with polysorbate 80 (1.5 and 2.0% w/v). These media were selected according to the NMS solubility results previously determined. The total test time was up to 3 hours and samples were withdrawn at determined time periods for drug quantitation (UV absorption spectrometry).

- **Bioavailability study**

The bioavailability study was assessed with six healthy volunteers of both sexes. After an overnight fast, the volunteers received a single oral dose (100 mg) of study formulation. Blood samples were collected before (zero time) and 30 minutes, 1, 2, 3, 4, 6, 8, 12 and 24 hours after the administration of NMS tablet. The blood samples were centrifuged and the plasma stored at –20°C. Plasma NMS concentrations were analyzed by HPLC system (Waters) equipped with a column C18 4 μm (3.9 x 125 mm) by a previously validated methodology. The mobile phase consisted of a mixture of acetonitrile:water:glacial acetic acid (45:54:1) at a flow rate of 1.0 mL/min. The injection volume was 20 μL and sodium diclofenac was the internal standard. The detection was by UV at 300 nm. The plasma treatment was by precipitation of plasmatic proteins with acetonitrile (1:1). The samples were centrifuged and the upper layer was injected into the liquid chromatograph. Pharmacokinetics parameters were obtained from the NMS plasmatic concentration versus time curves employing the software *WinNonlin Professional*. The relative fraction absorbed of the drug, in each time, was calculated according to Wagner-Nelson method, once the drug follows the one-compartment model.

- ***In vivo-in vitro* correlation establishment**

In order to define the dissolution test conditions, correlation analysis between relative fraction absorbed and *in vitro* dissolved percentage, obtained under different conditions, were performed. The *in vitro* results were paired with *in vivo* data obtained the double time of the former. The best correlation was assessed by linear regression and both correlation coefficient and slope were analyzed to support the choice of the dissolution condition.

Results and discussion

For equivalent concentrations (% w/v) of sodium lauryl sulfate and polysorbate 80 in water, the first provided always faster and more complete NMS dissolution, in both speeds evaluated. For all condition investigated, the dissolution of NMS was slower at 50 rpm. The dissolution profiles obtained with NaLS at 1.0; 1.5 and 2.0 % (w/v) and with polysorbate 80 at 1.5 and 2.0 % (w/v) were almost superposed with each other. Therefore, the higher surfactant concentrations can be considered unnecessary (more than 1.0 % for NaLS and than 1.5 % for polysorbate 80). The use of a surfactant in NMS dissolution studies is necessary to promote drug solubility, but it concentration should not be very high in order to became poor discriminating medium [2]. The selected medium presented both pH (6.45) and superficial tension (39.4 mN/m) within the physiological range.

Pharmacokinetics parameters calculated by the software for NMS tablets studied were: C_{max} = 5.6 μg/mL; t_{max} = 4.3 h; AUC_∞ = 82.3 h. μg/mL; $t_{1/2}$ = 6.4 h and K_e = 0.11 h^{-1}. Most of these parameters suggest a slower drug absorption when compared with respective values from literature for NMS tablets (C_{max} = 2.8-6.5 μg/mL; t_{max} = 1.2-2.7 h; AUC_∞ = 14.6-541 h. μg/mL; $t_{1/2}$ = 1.8-4.7 h) [3]. From the plots constructed between relative fraction absorbed versus amount dissolved, in different condition, the higher correlation coefficients obtained were with the media NaLS – 0.5 % at 50 and 75 rpm, and polysorbate 80 – 1.5 % at 75 rpm. The NaLS – 0.5 % at 75 rpm yielded a correlation with high slope, which indicate a less adequate relationship between the dissolution rate and the absorption kinetics process. The figures 1 and 2 illustrate correlations.

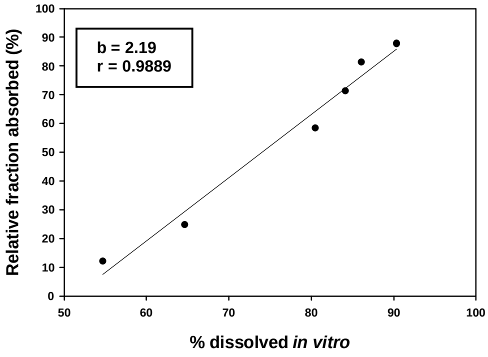

Figure 1- Relative fraction absorbed versus % dissolved of NMS in medium LSS – 0.5 %, at 50 rpm. (b = slope; r = correlation coefficient).

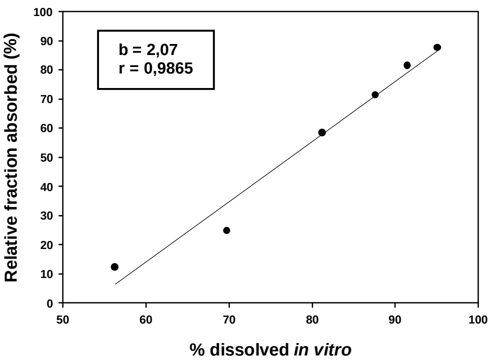

Figure 2- Relative fraction absorbed versus % dissolved of NMS in medium polysorbate 80 – 1.5 %, at 75 rpm. (b = slope; r = correlation coefficient).

For poorly water soluble drug products, dissolution testing at more than one time point for routine quality control is recommended [2]. So, we could suggest the following specifications for NMS tablets dissolution: medium polysorbate 80 (1.5 % - w/v) in water; volume of 1000 mL; apparatus 2, 75 rpm; Q_{15min} = 40 % and Q_{45min} = 75 % of labeled amount.

Conclusion

The best *IV-IV* correlation between relative fraction absorbed and NMS dissolved were obtained with LSS – 0.5 % (50 rpm) and Poli 80 – 1.5 % (75 rpm), both presenting correlation coefficient higher than 0.98 which demonstrates the relationship between the *in vitro* dissolution rate and *in vivo* absorption. Pharmacokinetics parameters computed for the studied formulation suggest a slower drug absorption than the respective values from literature for NMS tablets. Mild dissolution conditions furnished good *IV-IV* correlation for NMS tablets studied.

Acknowledgements

CAPES

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QC-05 PHARMACEUTICAL EQUIVALENCE OF LAMIVUDINE AND ZIDOVUDINE TABLETS

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Introduction

Since the discovery of the acquired immunodeficiency syndrome (AIDS) in 1981, considerable progress has been made in the development of anti-HIV agents. Zidovudine was one of the first 2'-3'-dideoxynucleosides to cause *in vitro* inhibition of HIV replication, by inhibiting the viral reverse transcriptase. Some trials showed that zidovudine results in clinical and immunological improvements and prolonged life in patients with AIDS or AIDS-related complex [1]. Lamivudine is other dideoxynucleoside analogue which undergoes intracellular phosphorylation to the putative active metabolite, lamivudine triphosphate. Combination antiretroviral drug is now generally considered preferable to monotherapy as first-line treatment for patients with HIV infections. Lamivudine administered in combination with zidovudine is now established as an effective agent for the treatment of antiretroviral drug-experience or – naive individuals with asymptomatic or symptomatic HIV diseases [2]. Although the association of lamivudine and zidovudine has been studied in terms of therapeutic activity and commercialized, there is no official pharmacopoeial monograph for determination of these drugs in tablets. A review of the literature indicated there were no reported dissolution or assay methods for a combination lamivudine plus zidovudine product. Few reports about analytical methods in biological fluids are available in the literature such as UV-spectrophotometry [3], HPLC [4,5,6]. The objective of the present study was to evaluate the pharmaceutical equivalence between five different commercial samples of 150+300mg lamivudine and zidovudine tablets available in the Brazilian market and compare with the reference product. The samples were evaluated by the following tests: aspect, average weight, hardness, friability, disintegration time, drug identification and assay, uniformity of dosage units and dissolution assay.

Experimental

- **Materials**

Lamivudine (LAM) and zidovudine (ZID) reference substances were kindly supplied by Laboratório Cristália. The tablets were claimed to contain 150 mg of lamivudine and 300 mg of zidovudine. Five samples (A, B, C, D and E) of different Brazilian pharmaceutical companies and the reference product (R) were evaluated. All other chemicals were analytical grade or HPLC grade.

- **Physical analysis**

The aspect, average weight, friability, hardness and disintegration tests were carried out according to the Brazilian Pharmacopoeia [7].

- **Uniformity of dosage units and assay**

The quantitative analysis were performed trough a validated high performance liquid chromatography (HPLC) method with UV detection at 270 nm. The chromatographic conditions were C_8 column (125 x 4mm); mobile phase: methanol and acetate buffer (50:50); flow rate: $1.0 mL.min^{-1}$. The HPLC system used consisted of a Shimadzu autoinjector LC-10AD pump, a variable detector SPD 10AD. Chromatograms were recorded and analyzed using a Class-VP® (Shimadzu) chromatographic data system. The samples were firstly diluted with hydrochloric acid 0.1N and after with the mobile phase. The injection volume was 20 µl.

- **Validation of HPLC method**

The method was validated by determination of the following characteristics [8]: linearity, range, accuracy, precision (repeatability and intermediate precision), specificity and robustness.

- **Dissolution test**

The dissolution medium used was 900 mL of water at 37 ± 1°C. Agitation was accomplished via the USP Apparatus 2 (paddles) at 50 rpm, during 30 minutes. Samples aliquots of 20 ml were taken, filtered through a 0.45 µm Whatman PVDF autovial syringeless filter, diluted with mobile phase and analyzed. The drug concentration in dissolution medium has been determined by HPLC method described above.

Results and discussion

According to the obtained results all the samples followed the specifications for the average weight, friability and hardness [7].

The HPLC method was linear (LAM: r = 0.9998; ZID: r = 0.9997), precise (R.S.D ≤ 2%) and accurate (5% of the nominal concentration), within the specific range (LAM: 24.0 - 36.0 µg/mL; ZID: 48.0 - 72.0 µg/mL).

Uniformity of dosage units results and corresponding assay are shown in Table 1**.**

Table 1 – Analysis of 150+300 mg lamivudine plus zidovudine in tablets by HPLC method.

Product		Assay (%)	Uniformity of dosage units (%)		
			Low	High	RSD
R	LAM	96.52	95.05	100.90	1.85
	ZID	96.26	96.50	101.70	1.69
A	LAM	98.85*	96.88	102.09	1.72
	ZID	102.23*	100.40	104.31	1.30
B	LAM	96.62*	92.52	102.40	3.10
	ZID	99.52*	94.29	101.23	2.45
C	LAM	96.80*	91.51	102.82	4.06
	ZID	100.78*	93.45	106.04	3.94
D	LAM	101.86*	94.40	100.76	2.29
	ZID	104.94*	97.27	102.79	1.93
E	LAM	93.24*	90.26	97.30	2.63
	ZID	95.81*	93.12	101.03	2.50
Limits		95.00-105.00	85.00	115.00	≤ 6

* in relation to the reference product.

The results of the *in vitro* study of the tablets containing lamivudine + zidovudine from different manufactures showed that only the product E was not equivalent to the reference.

The experimental values obtained for dissolution tests are present in Table 2.

Table 2 – Dissolution of lamivudine plus zidovudine tablets using Apparatus 2 (paddles) at 50 rpm and 900 ml of water.

Product		% Dissolved			
		Low	High	Mean	n
R	LAM	89.64	101.43	95.29	6
	ZID	87.09	93.57	90.02	
A	LAM	94.13	104.50	97.38	6
	ZID	91.72	97.96	95.61	
B	LAM	91.64	98.75	95.03	6
	ZID	87.72	95.81	91.55	
C	LAM	87.93	106.41	95.42	6
	ZID	84.50	96.89	89.94	
D	LAM	75.76	94.86	88.26	12
	ZID	77.50	94.21	88.70	
E	LAM	85.33	97.53	92.71	6
	ZID	84.68	99.17	94.07	
Limits	S1	≥ 80 %		≥ 80 %	6
	S2	≥ 60 %		≥ 80 %	12

Conclusion

The results demonstrated the importance of pharmaceutical equivalence for the generic products that can be interchangeable, for the therapeutic treatment. In the following steps it will be included the evaluation of the dissolution profile of these products.

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QC-06 EVALUATION OF DISSOLUTION PROFILE OF SOLID DOSAGE FORMS CONTAINING CHLORAMPHENICOL

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Introduction

The chloramphenicol is a natural antibiotic with a wide spectrum of antimicrobial activity [2].
The aim of this study was to evaluate the *in vitro* release (dissolution) of chloramphenicol from capsules, film-coated tablets and sugar-coated tablets, from different manufactures available in Brazil.

Experimental Methods

- **Dosage Forms**

Three different brands of 250 mg chloramphenicol capsules (A, B, C), two different brands of 250 mg chloramphenicol film-coated tablets (D, E) and three different brands of 250 mg chloramphenicol sugar-coated tablets (F, G, H) were tested.

- **Procedures**

The studies were performed by using Hanson Research apparatus. Samples were submitted to the dissolution test advocated by USP 24 (2000) [5] for chloramphenicol capsules (Table 1). Dissolution profiles were obtained on the same conditions. Samples (10 ml) were removed at 1, 5, 10, 20, 40 and 60 minutes. Aliquots of drug-free dissolution medium were added immediately to the dissolution vessel to keep the volume of the dissolution medium in the container constant during the test.

Table 1 – Conditions used for the dissolution test advocated by USP24 for chloramphenicol capsules.

Agitation system	Apparatus 1 (basket)
Stirring rate	100 rpm
Dissolution medium	HCl 0,01 N
Medium volume	900 ml
Detection (method)	Spectrophotometry UV (278 nm)
Sampling time	30 minutes

- **Statistical Analysis**

The methods investigated to compare profiles can be classified into three categories: ANOVA-based approaches, model-independent approaches, and model-dependent approaches.
Dissolution efficiency parameter (DE) was calculated from the percentage curves dissolved of drug substances *versus* time, through the ratio between the area on the curve (ASC) and the graphic total area (surface), and express it in percentage [3]. The ED values obtained were submitted to a statistical analysis using variance analysis (ANOVA) and model independent multivariate confidence region procedure (MSD/Tukey's tests) [1]. A second category of model independent methods, denoted pairwise procedures (difference factor, f_1 and similarity factor, f_2 was examined too.[4].

Results and discussion

Table 2 shows the individuals values of dissolution rate obtained for 250 mg capsules of chloramphenicol from three different manufacturers. According to the USP 24 (2000), the dissolution time of chloramphenicol capsules should be not less than 85% *(Q)* of the labeled amount of chloramphenicol dissolved in 30 minutes. The dissolution rate for the B and C brands did not comply with the USP requirements for amount of chloramphenicol (>85%). Product A was within the range of USP dissolution specification only after the second stage, S_2.

Table 2 – Percentage values dissolved of chloramphenicol shown in mean (M), standard-deviation (SD) and respective variation coefficients (VC), obtained for products A, B and C on the dissolution test specified by USP 24 (2000).

	Dissolution Test			
Vessels	*A*		*B*	*C*
	S_1	*S_2*		
1	82.83	93.07	39.39	2,05
2	84.66	89.86	31.75	1,93
3	82.06	76.10	38.78	1,95
4	77.94	79.01	65.07	1,81
5	92.77	84.82	69.35	2,09
6	71.21	83.75	68.74	3,15
M	83.17		52.18	2.16
SD	6.60		17.30	0.49
V.C.	7.94		33.15	22.84

The dissolution profile of the different brands (A, B, C, D, E, F, G and H) is shown Figure 1. Each data point represents the mean of 12 dosage forms. The mean percentage of chloramphenicol release for the different dosage forms (capsules, film-coated tablets and sugar-coated tablets) is presented in Figure 2.

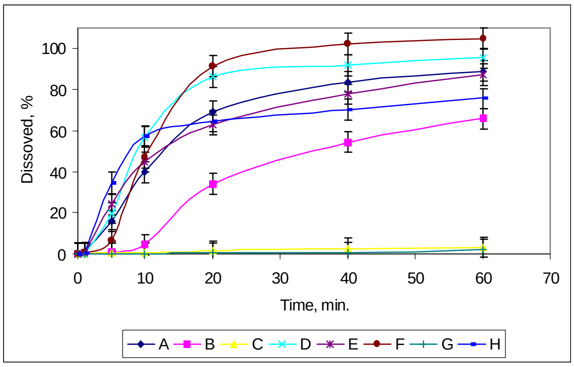

Figure 1 –Dissolution profile of chloramphenicol obtained for products A, B, C, D, E, F, G and H in medium HCL 0.01 N.

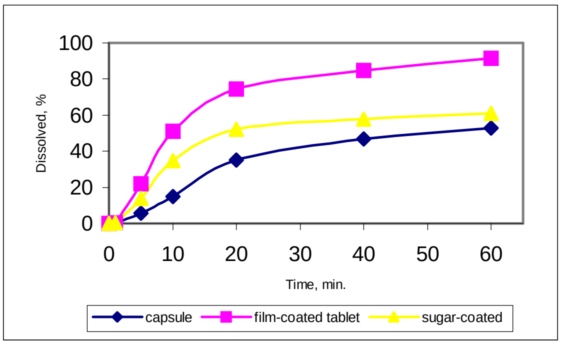

Figure 2 – Mean percentage of chloramphenicol release for the different dosage forms (capsules, film-coated tablets and sugar-coated tablets).

When capsules dosage forms were considered, the dissolution profile observed to all the brands were significantly different and the release-profile exhibited for C was unacceptable (Figure 1).

As seem in Figure 1, the products B, C and G showed dissolution values extremely low (less than 5% at 10 minutes).

When compared the mean dissolution profile exhibited for the capsules, film-coated tablets and sugar-coated tablets, the results presented for the capsules and sugar-coated tablets was significantly slower than the mean dissolution profile showed for the film-coated tablets (Figure 2). This can be ascribed to the time required for the mechanical destruction of the coated in sugar-coated tablets and hard gelatin capsules.

Based on ANOVA, statistically significant differences were found among the efficiency dissolution (DE) values for all the products studied (Table 3).

Table 3 – Variance Analysis (ANOVA) of DE values for pharmaceutical specialties A, B, C, D, E, F, G and H.

Variation Source	*Liberty Degrees*	*Square Sum*	*Medium Square*	*P Value*	*F **
Between Treatments	45428,3	7	6498,7	1,95 E-23	101,6
Within Treatments	2554,3	40	63,9		
Total	47982,7	47			

**Significant for P > 0,05*

In model-independent approaches pairwise procedures only product H showed dissolution profile similar for the reference product.

In instances where within batch variation is more than 15% CV, a multivariate model independent procedure is more suitable for dissolution profile comparison [4]. The acceptance criteria used for this test is wider than the pairwise procedure. The D and H products showed dissolution profile similar for the reference product.

Conclusion

From the results obtained in this work, it can be concluded that a significant variation exists in the *in vitro* release pattern of chloramphenicol from the solid dosage forms commercially available formulations in Brazil.

To the capsule dosage form (product A, B and C), the dissolution rate for the B and C products did not comply with the USP requirements for amount of chloramphenicol (>85%).

The products B, C and G showed dissolution values extremely low (less than 5% at 10 minutes).

Differences in biological availability of chloramphenicol in several dosage forms have beem reported. This can be ascribed to the physical characteristics of the drug and formulations techniques.

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QC-07 STABILITY OF ZIDOVUDINE (AZT) IN SOLID DISPERSIONS - ACCELERATED TESTS, THERMAL ANALYSIS AND KINETIC DECOMPOSITION OF COMMERCIAL PRODUCTS

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Introduction

Stability studies are designed to give insights into drug degradation mechanisms and expiration dating (shelf-life) estimations. Shelf-life is defined as the time required for a drug to decompose to 90% of its initial concentration at a specific temperature (T), relative humidity and light energy exposure [1].

Conventional stability testing includes prolonged storage at elevated temperatures and determination of the undergraded or degraded fraction of drug as a function of time [2]. The degradation rate under ambient conditions may then be estimated by extrapolation of the elevated temperature data using Arrhenius' equation [3]. In this method it spends a lot of time and, besides, the shelf-life prediction final results are uncertain due to temperature inconsistency in Arrhenius' equation. Consequently it would be desirable to have a rapid and less complicated method to perform stability testing [4].

The chemical and physical processes occur due to free energy variation, and the decomposition of drugs is usually accompanied by evolution of heat (exothermic process). Accordingly, another approach to do stability testing is by thermoanalytical methods that allow the determination of the evolved or absorbed heat in a relatively short time [3,4].

The aim of this work was to study the comparative stability of zidovudine (AZT) in products marketed by different laboratories. To provide a fair basis for comparison of the stability within a reasonable time scale, accelerated testing conditions are used that comply with those established by the International Conference on Harmonization (ICH). Thermal parameters of pharmaceutical formulations of zidovudine (100 mg) were determined by thermogravimetry (TG). The kinetic data, activation energy (E_a), reaction order (n) and frequency factor (*A*) were obtained by Osawa´s method and Arrhenius' equation.

Material and Methods

- **Samples**

Hard gelatin capsules containing 100 mg of zidovudine (AZT) from five Brazilian manufactures (here denominated by A, B, C, D and E) and reference product (R). All samples were acquired from Emílio Ribas Infectology Institute.

- **Accelerated testing**

The products were submitted to the same environmental conditions (40 ± 2°C and relative humidity at 75 ± 5%) for 180 days. The samples were withdrawn from the oven at pre-established times (30, 60, 90 and 180 days).

- **HPLC method**

The content of AZT was determined using a method modified and described by United States Pharmacopeia (USP) 24^{ed}. The capsules content (100 mg) were dissolved in methanol under 20 min sonication, after that diluted to 25 mL with mobile phase. This solution was then analyzed by high-performance liquid chromatography (Shimadzu, model LC-9A) with UV detection at 265 nm. The mobile phase consisted of methanol and phosphate buffer (pH=5) aqueous solution 40:60 (v/v), and the separation was carried out in a C_{18} column (Supelcosil LC-18, 250 x 4.0 mm, 5μm), at a flow rate of 1.0 $mL.min^{-1}$, under isocratic elution, with total time analysis of 6 min. The mobile phase and samples were filtered using a cellulose acetate membrane (HA 0.45 μm, Millipore).

- **Thermogravimetric studies**

In this study was used a TGA-50 system (Shimadzu) in a temperature range of 25-900°C using Pt crucibles with ~ 4 mg samples. The kinetic investigation of the drugs random degradation were obtained from thermogravimetric data by the application of Ozawa's method in which the slope of the plot of log (heating rate) *vs* 1/T gives the activation energy (E_a) of the process. In this study the heating rates used were: 2.5-5.0-7.5-10 and 15$^{o}C.min^{-1}$.

Results and Discussion

The HPLC method that was adapted for AZT's assay was sensitive and accurate with a relative deviation standard of 0.23% based on three measurements. A calibration curve was obtained (r = 0.9998) for a concentration of 1.2 - 4.8 μg/mL.

It was found that the six samples tested contain not less than 90% and not more than 110% of AZT as suggested by USP. The results of medium weight (mg/caps.) and content (%) for the AZT samples are showed in Table 1.

Table 1. Weight variation and content uniformity for the samples A - E and reference product (R).

Sample	Medium weight Mean (mg) RSD (mg)	Content per capsule (%)
A	182.7±4.5	101.7
B	172.7±5.8	101.5
C	167.7±3.8	96.8
D	240.3±7.3	99.5
E	168.1±7.1	95.4
R	231.7±2.8	99.7

In the accelerated stability study were not observed changes of color or other visible physical modifications apparent in the capsule's content. Also were not seen significant variations in the medium weight of the samples. Figure 1 shows the percentage of decrease of the content of AZT in accelerated study.

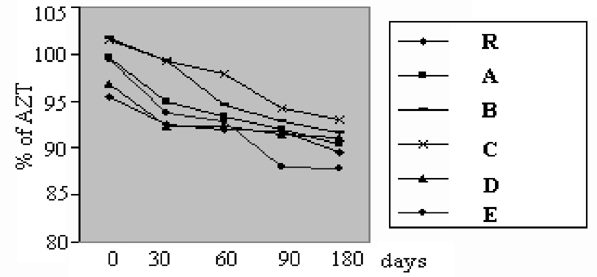

Figure 1. Variation of AZT contents (%) for R (reference product) and A - E samples (total of 180 days).

Figure 2 shows the TG curves of AZT (B sample) for the different heating rates. The results of TG indicated four stages of thermal decomposition in the temperature range 190-262, 262-320, 327-402 and 400-600°C.

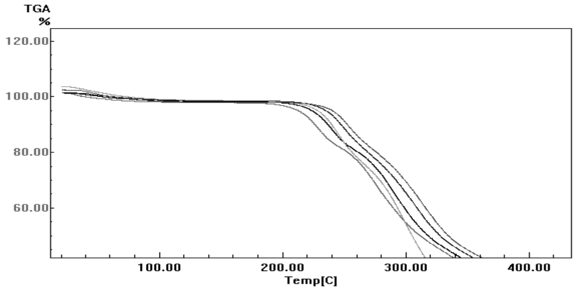

Figure 2. TG curves of AZT capsules (B sample) in nitrogen dynamic atmosphere and heating rates of 2.5-5.0-7.5-10 and $15^{o}C.min^{-1}$.

The obtained data from five TG curves were treated according to Ozawa's method in order to determine the activation energy ion stage in the temperature range of 150 to $250^{o}C$. The Figures 3 and 4 show the obtained plot and function Graphic G(x) of the inverse of temperature for AZT (B sample), which demonstrate a good correlation at the five heating rates. These results suggest a thermal decomposition process with reaction order zero and pre-exponential factor of 4.75×10^{13} min^{-1}.

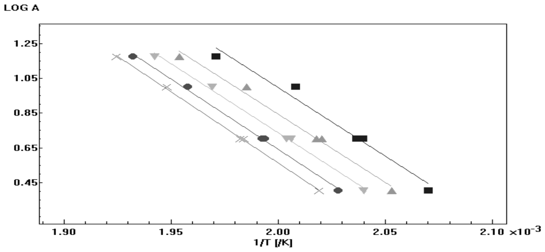

Figure 3. Osawa's plot for AZT capsules (B sample).

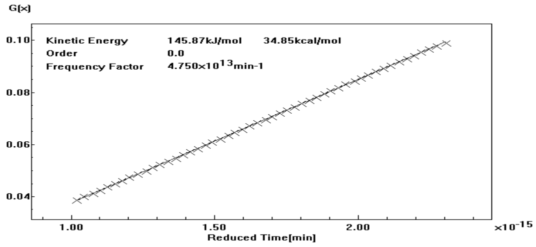

Figure 4. Function Graphic G(x) of the inverse of temperature for zidovudine capsules (B sample).

Stability parameters of the AZT samples determined by Osawa's method is showed in Table 2.

Table 2. Kinetic parameters of the AZT samples.

Sample	E_a (kJ mol^{-1})	Freq. Factor (t/min)	Temp. 5% loss (°C)
A	92	1.16×10^{8}	185-237
B	145	4.75×10^{13}	190-236
C	142	2.11×10^{13}	183-236
D	126	1.01×10^{12}	187-224
E	141	1.37×10^{13}	182-236
R	137	3.71×10^{12}	200-240

Conclusion

This study showed that thermoanalytical techniques have direct application in the evaluation of kinetic and determination of the thermodynamic parameters that can occur within a formulation. Preliminary dates evidence that thermal analysis is a potential technique to predict the shelf-life of drugs.

Acknowledgments

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QC-08 STABILITY OF EXPERIMENTAL BATCHES OF DIDANOSINE BUFFERED CHEWABLE TABLETS

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Introduction

2’,3’-Dideoxyinosine (didanosine, ddI) is a synthetic purine didesoxynucleoside, analogue of inosine, which shows high anti-retroviral activity [1]. The drug was approved by the Food and Drug Administration in 1991 for Human Immunodeficiency Virus (HIV) infected patients who are intolerant or resistant to Zidovudine therapy [2,3]. The ddI is available in Brazil as buffered chewable tablets (25, 50 or 100 mg) commercialized in polyethylene recipients with 60 tablets, produced by Bristol-Myers Squibb (Videx®). In Brazil, didanosine 100 mg tablets are also produced by official laboratories within the governmental AIDS program.

Objective

The purpose of this work was to investigate the stability of three experimental batches of didanosine 100 mg buffered chewable tablets manufactured by Ezequiel Dias Foundation (FUNED, the local official laboratory) in two types of packages (polyethylene recipients and aluminum strip), under three conditions (room temperature, 30°C/70% relative humidity and 45°C/85% relative humidity) [4,5,6].

Methodology

Three experimental batches of 100,000 units each were produced and packed in two sorts of containers. The batches were identified as: L1A, L1P, L2A, L2P, L3A and L3P, where A (aluminum strip) and P (polyethylene recipient) represent the packaging material. The batches were stored in three conditions and their testing frequency were:
Room temperature: 0, 1, 2, 3, 6, 9, 12 and 18 months.
Long-term condition (30ºC/70% relative humidity): 0, 3, 6, 9, 12 and 18 months.
Accelerated condition (45ºC/85% relative humidity): 0, 1, 2, 3, 4, 5 and 6 months.
The following physico-chemical tests were performed based on the general criteria of Brazilian Pharmacopoeia [7]: aspect, hardness (min. 3 Kgf), disintegration (max. 30 minutes in water) and friability (max. 1.5%).
The acceptance criteria for the content of water (max. 5% KF), the dissolution test (basket, water, 1000 mL, 100 rpm, 45 minutes; Q = 75%) and the HPLC assay (C8 column of 250 mm × 4.6 mm; mobile phase constituted by 1.4 mg/mL sodium acetate: methanol (68:32), pH adjusted to 6.5 with glacial acetic acid; flow: 1 mL/min.; injection volume: 20 µL; work concentration: 10 µg/mL; detection in 254 nm; 90% - 110% of the labeled amount) were based on the manufacturer specifications.

Results and discussion

- **Room temperature stability**

After 18 months, it was observed that the batches were in agreement with the specifications with maintenance of their physical characteristics. When in polyethylene recipient the content of water increased (e.g.: L3P: from 5.98% to 6.24%) and decreased when in aluminum film (e.g.: L1A: from 6.45% to 4.31%). Hardness was shown independent of the packaging and storage conditions. The results oscillated between 4.5 and 10.5 Kgf. Friability increased with time of storage (from 0.40 to 1.39%), but was independent on the packaging. Disintegration, dissolution and assay were according with the requirements for both packaging and store conditions.

- **Long-term stability (30ºC/70% relative humidity)**

After 18 months, the batches did not present any change of aspect. The humidity (around 6%) and hardness (oscillated from 4.25 to 8.82 Kgf) showed to be independent up the packaging and storage conditions. Friability had tendency to increase with storage time, independent on the packaging (from 0.40 to 1.49%). Disintegration test accomplished the requirements, while the dissolution test presented problems of release after 12 months, e.g.: L1A (65.74%); L1P (64.58%); L3P (75.12%). The content of didanosine on the three batches were maintained according to the specifications (90% - 110% of the labeled amount) independent on the packaging and storage conditions (results between 91.8% and 102.0% of the labeled amount).

- **Accelerated stability (45ºC/85% relative humidity)**

After 6 months, no change in aspect was observed. The humidity (around 6%) and hardness (from 4.56 to

7.73 Kgf) were independent up the packaging and storage conditions. Friability had a tendency to increase with time and was independent up the packaging (from 0.40 to 1.38%). The disintegration time increased mainly in the aluminum strip packaging, and did not comply with requirements after, a certain period (e.g.: L1A: 4 months, L1P: 5 months, L3A: 5 months). The tablets packaged in aluminum strip presented greater difficult of release than those in polyethylene, after one month (e.g.: L1A: 36.67%, L1P: 58.47%, L2A: 69.26%, L2P: 94.91%, L3A: 59.76%, L3P: 76.24%). The didanosine assay complied with the requirements (values between 91.8% and 102.0% of the labeled amount), independent up the packaging and storage conditions.

Conclusion

The results demonstrated the high stability of didanosine tablets produced by FUNED during 18 months in room temperature, independent upon the packaging (polyethylene recipient or aluminum strip). However, problems related to the disintegration and dissolution occurred when stored at 30ºC under 70% relative humidity or at 45ºC under 85% relative humidity. In such conditions, polyethylene recipient seems to offer better protection to the tablets.

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QC-09 STUDY OF THE THERMAL STABILITY OF GLIBENCLAMIDE THROUGH ISOTHERMAL ANALYSIS AND ACCELERATED DECOMPOSITION IN OVEN

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Introduction

The development of new pharmaceuticals formulations requires the choice of appropriate excipients. It is very important to ensure physical and chemical stability of the product. The traditional concept of excipient as any component other than the active substance has undergone a substantial evolution from an "inert" and cheap vehicle to an essential constituent of the formulation .

Excipients are employed to carry out different functions that may be grouped into three categories, according to whether they influence stability, release and absorption of the active principle or manufacturability during the manufacturing process phase.

Hence, there is the need to forecast incompatibilities between drug and excipients during the pre-formulation steps.

The physicochemical properties of pharmaceutical powders have direct influence the quality and the stability of the dosage form. The real time and the accelerated storage studies are very expensive and may be misleading because it will only be possible to verify some incompatibility between active principle and excipient a long time after the beginning studies.

In this paper we have compared accelerated storage in oven studies of glibenclamide with that of isothermal curves, since this techniques has increased its use for quick evaluation of the possible incompatibility through the comparison of thermal analytical curves of pure substance with the curve obtained for binary mixture.

Materials and Methods

All the materials were obtained from “Instituto de Tecnologia em Fármacos – Far-Manguinhos “of Fiocruz.

The glibenclamide tablets were prepared homogenizing the active principle and the following excipients: microcrystalline cellulose , magnesium stearate and sodium croscamelose. All components were passed through a fine-sieve of 65 mesh and homogenized in a Lawes mixer, “v” model NI50FPZI, during 15 minutes. This mixtures were compressed with puncture 7.0 mm in a Lawes-2000/10 PSC compression machine.

Preliminary studies using thermogravimetric analysis were carried out in a thermal balance Perkin-Elmer model TGA-7 under nitrogen flow of 20 mL/min. heating rate of 10°C/ min. from room temperature up to 300°C and then 300°C to 600°C using a heating rate of 20°C/ min., with samples of approximately 10 mg weighed into sealed hermetically aluminum pans. These preliminary studies were carried out with drug , pharmaceutical excipients and binary mixtures and gave the behaviour of the samples at high temperatures.

The isothermal TG studies were carried out using a Perkin-Elmer DSC-7 differencial scanning calorimeter under nitrogen flow of 20mL/min,. heating rate of 10°C/min. from 30°C to 80°C and the isothermal curves were obtained at temperatures of 80, 90, 100 and 110 °C during 245 minutes.

The glibenclamide tablets were placed in an oven with constant temperature at 40^{o}C , and humidity of 75%, and 50^{o}C with humidity of 90%, and were analyzed in the UV with intervals of 30 days during six months.

The rate constants of the thermal decomposition were determined for the isothermal method based in the Arrhenius law.

Results and Discussion

The preliminary TG studies on the active principle, excipients and binary mixture showed, as expected , that the behaviour of the binary mixture is altered on mixing all components.

These data enabled us to establish the parameters for the isothermal studies.

Table 1 and 2 show the results of the accelerated decomposition in oven.

Table 1 - Temperature 40^oC with humidity of 75%

Time [days]	0	30	60	90	120	150	180
Concentracions of drug *[mg/tablet]*	*5.00*	*4.97*	*4.93*	*4.91*	*4.93*	*4.88*	*4.90*

Table 2 - Temperature 50^oC with humidity of 90%

Time [days]	0	30	60	90	120	150	180
Concentracions of drug *[mg/tablet]*	*4.99*	*4.98*	*4.91*	*4.93*	*4.89*	*4.78*	*4.77*

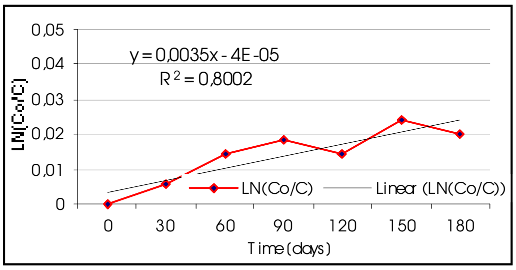

Figure 1 – Linear Adjustment of the LN of glibenclamide concentrations in tablets as a function of time, at 40^oC

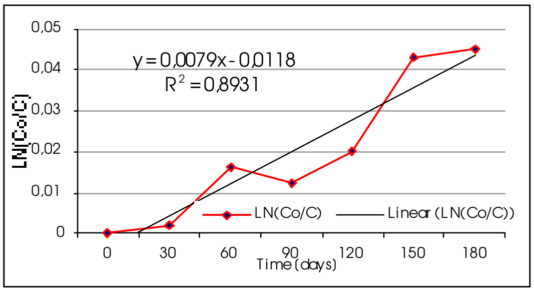

Figure 2 – Linear Adjustment of the LN of glibenclamide concentrations in tablets as a function of time, at 50^oC

The rate constants for the accelerated decomposition in oven for the glibenclamide tablet are: $K_{40}=3.84x10^{-4}$ and $K_{50}=8.52x10^{-4}$ The isothermal decomposition method gave different results from those of the accelerated decomposition method.

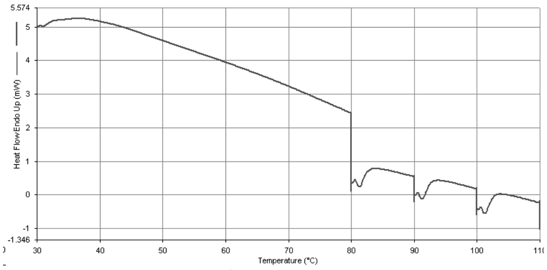

Figure 3 - Isothermal of the mixture binary

However the isothermal data don't confirm the good stability of these formulations. We believe this analysis cannot be a sufficient conditions will prove incompatibility between excipients and drug, because the physicochemical stability of these formulations have already been proved at room temperature tests. Hence, it is clear that any method of early detection should be used, even if such a method is only able to screen out the most problematic formulations.

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QC-10 VALUATION OF THE ISOTHERMAL TECHNIQUE AS A TOOL TO STUDY CHEMICAL STABILITY AT SHELF LIFE

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Introduction

Drug and the excipients should be compatible and stable to assure the quality, safety and efficacious of the pharmaceutical product. Thermal analysis has been used to investigate drug-excipient interactions and, in consequence, their compatibility.

Conventional stability testing includes prolonged storage at elevated temperatures and determination of the undegraded or degraded fraction of drug as a function of time.

Techniques conventional stability testing are time consuming and, moreover, suffer from the uncertainty of the final shelf life prediction due to temperature dependent inconsistencies in the Arrhenius relationship. Consequently it would be desirable to have a rapid and less complicated method to perform stability testing.

Than, in order to evaluate the application of changes in curves isothermal like prevision of interactions, or possible incompatibility, that occurs between drug and excipient during storage at room temperature, we compared the changes in curves isothermal with results of Propranolol's stability at shelf live. We acknowledge that the presence of a physical or chemical interaction does not necessarily indicate incompatibility, however the aim of our study is evaluation the use isothermal techniques as another a tool for to preview stability of pharmaceuticals products at shelf life.

Materials and Methods

- **Materials**

The Propranolol Hydrochloride was used as a model drug. As excipients were used Microcrystalline Cellulose 102, Magnesium Stearate and Sodium Starch Glicolate. All materials were donated by "Instituto de Tecnologia e Fármacos" – Far-Manguinhos of the Fiocruz.

The formulation of the Propranolol tablets was elaborated with following composition: Propranolol Hydrochloride drug 50%, Microcrystalline Cellulose 102, Magnesium Stearate and Sodium Starch Glicolate. The binary mixture of the Propranolol and excipients was prepared in quantitative proportions of the formulations for sieving with 65 mesh, collected porcelain grail by 10 minutes until the complete homogenized and compressed with puncture 7.5 mm.

Methods

- **Isothermal TG**

The isothermal curves of the binary mixtures was obtained in a Perkin-Elmer DSC-7 Differential Scanning Calorimeter under nitrogen flow of 20mL/min, heating rate of 10°C/min from ambient up to 70°C, using a mass of 8,00mg. The TG isothermal curves was obtained in temperatures of 70, 90, 110 and 130^{o}C for period of 240 minutes.

- **Conventional Stability Testing – Shelf Life**

The tablets were placed in stove with temperature constant at 30^{o}C, with constant humidify 70%. The concentration of sample were analyzed in HPLC with interval of in three months intervals, during a year. Each analysis was repeated three time with different tablets. The results is average of the three analysis.

- **Calculations Methods**

From the reaction scheme

$$A + B \rightarrow \text{products}$$

a kinetic equation were deduced, where *A* is Propranolol drug and *B* is all excipients and elementary Second order reaction was assumed.

$$\frac{C_A}{C_{Ao}} = \frac{1}{1 + k.t.C_{Bo}}$$

were C_{Ao} is Propranolol initial concentration, C_A Propranolol concentration in time t, C_{Bo} is excipients initial concentration and k is specific velocity of the reaction.

A plot of the evolved $(C_{Ao}-C_A)/C_A$ versus the $t.C_{Bo}$ showed a linear relationship. The Arrhenius equations were used to esteem degradation rate (k) and by extrapolation of the elevated temperature data this equations was done to esteem degradation rate (k) in ambient temperature.

Results and Conclusion

Isothermal data were compared with shelf life data in order to evaluate the possibility close estimate chemical stability and excipient compatibility of solid drug. The degradation rate was calculated for shelf life ($k_{30}=1.17x10^{-2}$) (Fig.1) and for isothermal data 70, 90, 110 and 130°C ($k_{70}=1.93x10^{-3}$, $k_{90}=2.01x10^{-3}$, $k_{110}=9.07x10^{-3}$, $k_{130}=2.01x10^{-2}$) (Tab. 1).

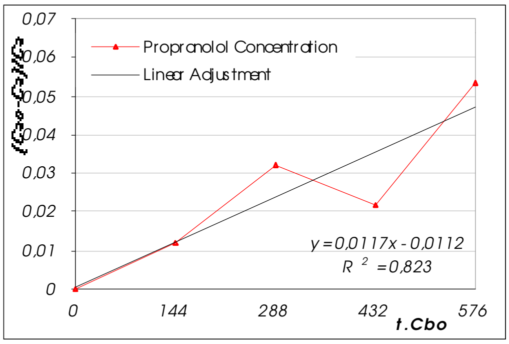

Figure 1 – Linear Adjustment of the Propranolol concentrations in tablets by time in shelf life.

Through linear adjustment of Arrhenius equation

$$k = k_0 \exp(\frac{-E}{R.T})$$

$$Lnk = Lnk_0 - \frac{E}{R}.\frac{1}{T}$$

were calculated the frequency factor ($k_0=3.42x10^{6}$) and the activation energy of reaction ($E=11.600cal/gmol$) (Table 1). Hence, the rate degradations in room temperature were calculated using this parameters in Arrhenius equations ($k_{30}=1.467x10^{-2}$).

Table 1 - Linear Adjustment of Arrhenius equation

Temperature [°C]	70	90	110	130
Rate Degradations (k)	$1.93x10^{-3}$	$2.01x10^{-3}$	$9.07x10^{-3}$	$2.01x10^{-2}$
T^{-1} [K^{-1}]	$2.915x10^{-3}$	$2.755x10^{-3}$	$2.611x10^{-3}$	$2.481x10^{-3}$
Ln (k)	-6.250	-6.210	-4.703	-3.907

The results shown a small discrepancy in rate degradations of the reaction between results both methods. The discrepancy could be due to some interaction between drug and excipient induced solely by the temperature increase during studies of isothermal curves and not preexistent in the mixtures stored in usual conditions.

It is possible, also, that the interaction between drug and excipients is so slow at room temperature that it is negligible but, on increasing the temperature, the kinetics of the reaction increase and the physicochemical properties of the components are changed.

Although, we believe that the small discrepancy in value of the degradation rates, cannot be a condition sufficient to prove compatibility between drug and excipient during storage at shelf live. Hence, it is yet clear to other tools must be used to improve the preview of interactions physical and chemical between drug and excipients at room temperature.

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QC-11 PURITY ASSAY OF SULFAMETHOXAZOLE RAW MATERIALS BY MEANS OF DSC-PHOTOVISUAL AND DISSOLUTION

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Introduction

The analysis of raw materials used in the pharmaceutical industry disposes of analysis methods with high costs and low identification and quantification sensibility.

The thermal analysis can be employed in purity determination [1], compatibility and stability studies [2,3], polymorphism determination [1-3] in pharmaceutical drugs and characterization of excipients and pharmaceutical formulations.

The solubility studies using dissolution technique can be used as procedure of quality control of pharmaceuticals raw materials into the pharmaceutical equivalence studies.

The implementation of the thermal analysis and solubility studies can be used in the selection of the raw materials and their respective suppliers.

The present work has characterized the sulfamethoxazole raw materials by means of DSC-photovisual and dissolution.

Experimental

- **Materials**

The sulfamethoxazole batches were supplied by distributors Dinalab, Palmares-Ervy and Ciel imported from China E03980698 and E15940399 and Germany E01110499, respectively. They were obtained at the Laboratory of Pharmaceutical Technology of the Federal University of Paraíba (LTF-UFPB).

- **Storage of the samples**

The samples were stored in amber flask at 25ºC up to the calorimetric and dissolution analyses.

- **Infrared analysis**

The sulfamethoxazole batch were analysed Fourier Transformer Infrared Spectrometer Bomen-Michelson apparatus, model MB 102, range 2000 and 400 cm-1, using KBr pellets.

- **Purity study**

The DSC apparatus was calibrated through the melting points of indium (156.6ºC ± 0.2) and zinc (419.6ºC ± 0.3) standards, under the same conditions of the samples. The DSC curves of the sulfamethoxazole batch were obtained in Shimadzu apparatus, model DSC-50, coupled to a Sanyo camera, model VCC-D520, connected to the Olympus microscopy, heating rate of 10.0°C/min., nitrogen atmosphere of 50.0 ml/min, up to 500.0°C. The samples were put in hermetically sealed aluminum pan and sample mass 2.0 mg.

Solubility profile in dissolutor

The solubility of the 3 sulfamethoxazole batches were obtained in VANKEL dissolution test station, model VK 7010, containing 8 vessels and coupled to VARIAN ultraviolet apparatus, model Cary 50 UV-Vis. The dissolution method was used from the American pharmacopoeia [5]. The analysis were accomplished in quadruplicate and collected at times: 5, 10, 20, 40 and 60 minutes. The calibration curve was realized with sulfamethoxazole USP standard.

The dissolution constants were accomplished with the Kitazawa model [4].

$$\ln w^{\infty}/(w^{\infty}-w_0) = k't$$

where: w^{∞}: amount of drug in solution at infinite time; $(w^{\infty}-w_0)$: amount of undissolved drug; k': dissolution constant; t: time in question.

- **Statistical Analysis**

The sulfamethoxazole batch submitted to the purity studies were appraised for the average and standard deviation of the melting point in DSC curves and the concentration in dissolution profile.

Results and Discussion

- **Infrared Analysis**

The infrared analysis showed similar absorption bands among the 3 batches analysed in the range from 1600 to 1500 cm-1 characterizing the aromatic bond C=C, bands in 1015 cm-1 characterizing the bond C-S. The infrared analysis did not get to differentiate the 3 sulfamethoxazole batches.

- **Purity study**

The DSC curves of the batch E03980698 presented melting point at (170.0 ± 0.20ºC), E15940399 (170.1 ± 0.28ºC) and E01110499 (170.0 ± 0.15ºC). The calorimetric data allowed to determine the purity of the raw materials 99,04%; 99,40% and 99,30%, respectively.

- **DSC-photovisual**

The comparison of the melting processes by means of DSC-photovisual demonstrated that the sulfamethoxazole batches present melting point with different coalescence rate and confirmed the purity degree of the lots. The batches E15940399 and E01110499 presented more limpid and transparency solutions than batch E03980698 after coalescence.

- **Dissolution profile**

The dissolution profile of the raw materials revealed great differences among the studied lots. The lots E15940399 and E01110499 presented smaller dissolution percentile, about 20%, when compared to the lot E03980698 that dissolved about 100% in 20 minutes.

The polymorphs presence, soluble organic pollutants originated during the synthesis route or decomposition products can be contributing for the high dissolution value for the lot sulfamethoxazole E03980698 (Figure 1).

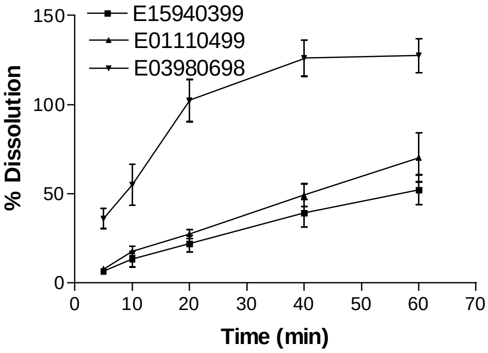

Figure 1. Dissolution profiles of the sulfamethoxazole batches

- **Dissolution Constants**

The Kitazawa model showed that the batches E01110499 and E15940399 presented similar dissolution constants amongst themselves and totally different from the batch E03980698. The dissolution constants revealed that the sulfamethoxazole batch E03980698 presented discrepancy in the dissolution constant in relation to the others (Figure 2).

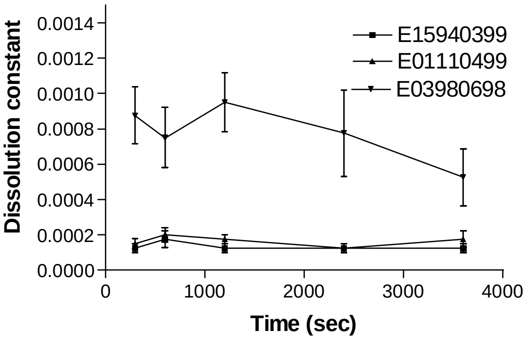

Figure 2. Dissolution constants profiles of the sulfamethoxazole batches

Conclusion

The infrared data and DSC revealed similarities among the sulfamethoxazole batches, however DSC-photovisual can be possible to differentiate them. The dissolution constants from Kitazawa equation demonstrated a powerful tool for the studies of sulfamethoxazole purity in the obtaining of standardized raw material guaranteeing the pharmaceutical equivalence of the medicines.

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QC-12 THERMAL AND DISSOLUTION STUDIES OF RANITIDINE PRE-FORMULATED AND FORMULATED

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Introduction

The new methods for the development of pharmaceutical formulations require previous knowledge of the physical-chemical properties of the drug and excipients, and analytical instrumentation with which results can be obtained rapidly and simply. The thermal characterization of the formulations affords the first parameters in compatibility [1].

The kinetic studies of dissolution appear as tool to detect the influence of critical variables of the technological process as binder effect, of the mixture, excipient type, and in the correlation studies *in vivo / in vitro* in solid pharmaceutical forms [2].

The implementation of the thermal analysis and kinetic studies of dissolution appears as comparative analytical method, producing fast and reproducible results that can be used in the selection of formulations with pharmaceutical equivalence candidates to the bioequivalence studies.

The present work proposes evaluation of the pharmaceutical equivalence of ranitidine pre-formulated and formulated by means of calorimetric studies and dissolution kinetics.

Experimental

- **Materials**

The ranitidine drug, formulated A and the excipients: diluent, disintegrant, adsorbent and lubricant were supplied by the Palmares distributor and manufactures at the Laboratório de Tecnologia Farmacêutica of the Universidade Federal da Paraíba (LTF-UFPB). Tablets B and C (formulated reference) were acquired in the local drugstore without a described formula.

- **Preparation of the Pre-Formulated**

The samples were prepared by two way: 1) no stressed and 2) previous stressed. 1) They were sifted through a 100 mesh sieve, collected in porcelain grail, homogenized for 10 minutes and conditioned in amber flask. 2) The samples with previous stress were submitted to compression force in 9mm punches.

The pre-formulated of ranitidine were prepared in the following proportions: Ranitidine + diluent unstressed (1:1, 6:4, 7:3, 8:2, and 9:1) and previous stress (1:1, 6:4, 7:3, 8:2) received 3.15, 3.10, 3.05 and 3.05 tons in compressed; Ranitidine + disintegrant no stressed (90:10, 95:05, 97:03, 98:02), Ranitidine + disintegrant stressed (85:15, 90:10, 95:05, and 98:02) 2.95 tons in compressed, ranitidine+adsorbent no stressed (99:01, 99.3:0.7, 99.7:03, 99.9:0.1) and Ranitidine + lubricant no stressed (95:05, 98:02, 99:01, 99.3:0.7, 99.5:0.5).

- **Compatibility study**

The DTA apparatus was calibrated through the melting points of indium (156.9ºC ± 0.2) and zinc (419.8ºC ± 0.3) standard under the same conditions as for the samples. The differential thermal curves of ranitidine drug, binary mixtures with previous stresses and no stressed and tablets A, B and C were carried out by means of DTA apparatus, model DTA-50, heating rate of 10.0°C/min., nitrogen atmosphere of 50.0 ml/min, up to 500.0°C. The samples were put hermetically sealed in aluminium pan. The samples mass was 6.50 mg.

- **Dissolution profile and kinetic**

The dissolution profile of tablets A, B and C were obtained in a VANKEL dissolution test station, model VK 7010, containing 8 vessels, coupled to a VARIAN spectrophotometer. The dissolution method was specified by the American pharmacopeial (USP XXIV). The times of collections for the 6 tablets was accomplished in the times: 5, 10, 15, 20, 25, 30, 45, 60 and 75 minutes.

The calibration curve was accomplished with the ranitidine USP standard.

The kinetics of dissolution was determined with the *f1 x f2* parameters and the model of Kitazawa [3].

- **Statistical Analysis**

Pre-formulated submitted to the compatibility study no stressed and stresses were appraised by the average and standard deviation of the melting point.

Average and standard deviation of the concentrations were calculated in the dissolution profile of the tablets A, B and C (reference).

Results and Discussion

- **Compatibility study**

The DTA curves of the pre-formulated no stressed presented phase transitions at temperatures: ranitidine+adsorbent (144.38 ± 0.36ºC), ranitidine+ lubricant (144.66 ± 0.41ºC). The curves DTA of the pre-formulated of ranitidine+diluent and ranitidine+ disintegrant with previous stressed showed small displacement of the melting point of (144.78 ± 0.58ºC) for (146.03 ± 0.32ºC) and (144.54±0.63ºC) for (144.71± 0.35ºC), respectively, in all proportions studied. The tablets A (145.06 ± 0.38ºC), B (145.26 ± 0.51ºC) and C (147.16 ± 0.43ºC) (reference) did not present displacement of the melting point in relation to ranitidine drug (144.35 ± 0.38ºC).

- **Dissolution profile**

The dissolution profile of tablet A and C showed superposition among themselves, however tablet B presented smaller liberation rate in relation to the tablets A and C (Figure 1).

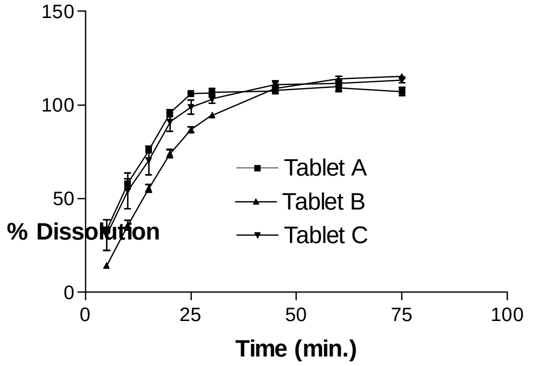

Figure 1. Dissolution profile of the ranitidine tablets.

The f1 x f2 analysis from the dissolution profile showed that tablet A and C confirmed the similarity between tablets A and C, while tablet B did not present pharmaceutical equivalence (Table 1).

Table 1. Analysis of f1 x f2 to the tablets A and B compared to tablet C.

Time (min)	Tablet A		Tablet B	
	F1	F2	F1	F2
5	4.58	88.22	53.24	39.36
10	3.47	87.49	40.88	37.99
15	4.05	80.26	31.96	38.96
20	3.30	81.09	27.01	38.87
25	3.92	74.37	22.74	39.98
30	3.51	75.21	19.39	41.36
45	3.70	72.93	15.91	42.99
60	3.71	72.23	13.58	44.39
75	3.91	70.37	11.87	45.63

- **Dissolution Constants**

The dissolution constants showed different liberation rates (Figure 2). The tablets A and C presented similar profile, which were meaningfully different from tablet B. The kinetic parameters obtained from Kitazawa equation revealed a rigorous tool for the pharmaceutical equivalence studies, according to results from f_1 and f_2 parameters.

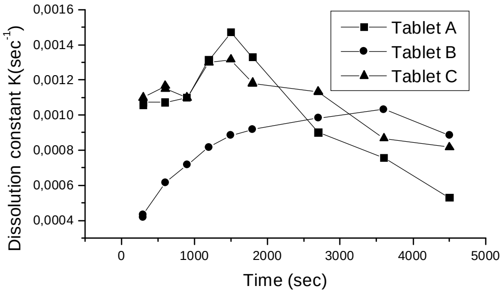

Figure 2. Dissolution kinetic of the ranitidine tablets.

Conclusion

The DTA data revealed compatibility among the tablets A, B and C. These data demonstrated also that the stress did not alter the thermal profile in the formulations. The dissolution kinetics showed great importance to the pharmaceutical equivalence studies.

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QC-13 THERMAL CHARACTERISATION AND SOLUBILITY OF SOME CHLORPROPAMIDE RAW MATERIALS

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Introduction

Chlorpropamide is poorly water soluble and has a show dissolution rate. Significant therapeutic differences have been observed between the different forms, which supports the fact that the low solubility and poor rate of solution of the drug are important factors limiting its use in the treatment of several diseases [1-3]. The form differ with respect to their infrared (IR) and differential scanning calorimeter (DSC). During this generic development study, raw materials samples were studied in order to determine whether they contained form [4].
The present work proposes to evaluate the chlorpropamide form present in raw materials for thermal analysis, infrared spectra and "dissolution".

Experimental methods

- **Samples**

The chlorpropamide powders were obtained from three suppliers (lot. QDC/CP136, Holland), (lot.CP/EXP-23, China) and (lot. CP/EXP-22, China).

- **Infrared spectrophotometer**

The infrared spectrum were recorded on Fourier transformed infrared spectrophotometer (Bomem, MB100 michelson series) over a range of 600-4000 cm^{-1}. The KBr disk technique was used to measure the IR spectra of the raw materials.

- **DSC**

The DSC curves of the chlorpropamide samples were obtained with a Shimadzu calorimeter, model DSC-50 coupled to the photovisual system constituted by Olympus microscope connected a Sanyo camera, model VCC-D520, with nitrogen atmosphere of 50 mL.min^{-1}, heating rate of 5 °C.min^{-1}, up to the temperature of 500°C. The samples were packed in aluminium cell with mass about 2.0 mg. The images were captured by DSC coupled to the photovisual system in similar temperature and time compared to the conventional DSC.

- **Powder "Dissolution"**

Powder dissolved was measured using Dissolution Testing Station (Vankel), Method 2, paddle, of the USP 23 (4). The paddle was rotated at 50 rpm and samples were taken at 2, 4, 8, 15, 30 e 60 min. Transfer an accurately weighed portion of the powder, equivalent to about 250 mg in 10 mL of suspension chlorpropamide. The concentration of dissolved powder was calculated from the UV absorbency values obtained at 230 nm, results are the mean of six determinations.

- **Results and discussion**

According to Himmelreich et al. (1) the IR absorption spectrum show differences in the detailed shape and intensities of some of the large absorption bands. Specifically, the carbonyl (carbamate) stretching frequency (1700-1730 cm^{-1}) and –NH stretching frequency (3340-3410 cm^{-1}) were different in each form and were used to identify the three polymorphs. Lot. QDC/CP136 showed –NH stretching frequency 3339 cm^{-1} and carbamate stretching frequency 1709 cm^{-1}. However, the lots CP/EXP-22 and CP/EXP-23 presented NH stretching frequency 3354 and 3350 cm^{-1} and carbamate stretching frequency 1736 and 1701 cm^{-1} respectively.

DSC curves of the three samples show small different thermal behavior in the range 126 – 129°C. Lot. QDC/CP136 presented an endothermal peak corresponding to the melting point at temperature of 129.8°C and reaction heat – 89.8 kj/kg, CP/EXP-22 with melting point at 129.6°C and reaction heat -149.6 kj/kg and 129.6 kj/kg with melting point at 129.6°C and reaction heat of – 91.61kcal/kg (Figure 1). A second peak before melting peak presented differences among them. Such fact can be due the presence of impurity or polymorph.

Data analysis obtained from DSC coupled to the photovisual system showed different melting processes among the lots Lot. QDC/CP-136 (Figure 2 – pictures A, B and C) underwent alteration in volume and physical change at 129°C, corresponding to the melting point (pictures B), lot. CP/EXP-22 (pictures D, E and F) exhibited a small volume expansion around 143ºC (picture C) and lot. CP/EXP-23 reveal a thermal behavior well defined in its melting point (picture H).

The comparison of samples from three different lots revealed the value of photovisual system in the quality control of the raw material.

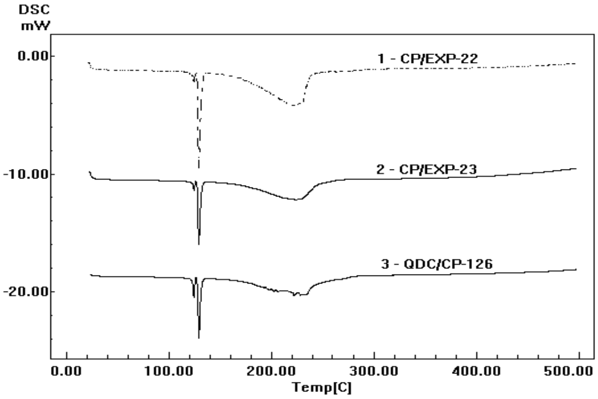

Figure 1. DSC curve of three chlorpropamide lots.

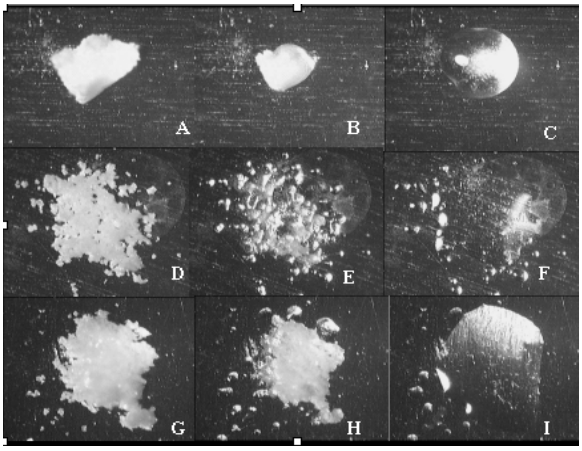

Figure 2. Pictures of three chlorpropamide lots obtained by DSC coupled to the photovisual system. Lot. QDC/CP136: pictures A (room temperature), B (129ºC) and C (143ºC), Lot. CP/EX-22: pictures D (room temperature), E (129ºC) and F (143ºC), Lot. CP/EX-23: pictures G (room temperature), H (129ºC) and I (143ºC).

The powder "dissolution" profiles of raw materials are given in figure 3. All the samples dissolved slowly and at completion of the dissolution test suspended particles were visible in the dissolution medium. The dissolution of Lot QDC/CP136, however, was slightly better than that of CP/EXP-22 and CP/EXP-23 (Fig.1). This could be a result of the different raw material solubility among lots. Some investigators had shown that kneading poorly soluble drug could improve the dissolution of the drug with differences median particle diameters.

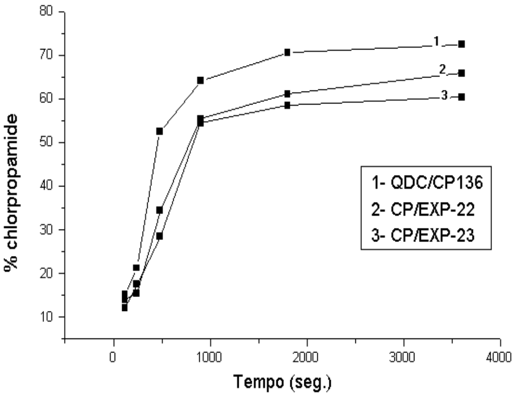

Figure 3. Dissolution profiles of three chlorpropamide lots.

Conclusion

Data analysis by thermal analysis, infrared and dissolution test confirmed differences among chlorpropamide lots.

Acknowledgments

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QC-14 STUDY OF THE *Calendula officinalis* L. (Asteraceae) STABILITY IN POLYPROPYLENE PACKAGING

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Introduction

Calendula officinalis L. (Asteraceae) is an annual herb native to the Mediterranean region. It is cultivated for ornamental and medicinal purposes in Europe and America. In Brazil, *C. officinalis* is commonly called “maravilha”and “flor de morto” and it has been considered to be a decorative plant.

The plant has been employed for a long time in folk therapy. More than 35 properties have been attributed to the decoctions and tintures from the flowers, e. g. choleretic, antiinflammatory, analgesic, antitumoral, antiulcer, bactericide, diuretic, tonic and the healting of wounds and skin eruptions. Antibacterial and antiinflammatory actions, among others, have been confirmed by experimental pharmacology. In the last years attention has been drawn by the Brazilian authorities upon its use in traditional medicine.

Calendula officinalis consists of a complex pharmaceutical material which is extremely important for the population. For this reason it is necessary to determine its real time validity, the best storage conditions and suitable packaging. This aims at giving guarantee to the level of the various classes of chemical compounds with distinct pharmacological activities, once small pachets are freely available for the consumer.

The polypropylene plastic bag is usually used as a container to the stabilized medicine plants. The Code of Federal Regulation recommends that the packets which are used to prevent pharmaceutical products from deteriorating and contaminating must characterize themselves as a system which can guarantee the quality of the final product. These packets must neither react nor allow any exchange with the external environment.

Several external factors such as temperature, humity, light and transport may interfere in the final quality of the pharmaceutical products. The pharmacies and the pharmaceutical industry have been making a great effort to search for the parameters which are able to keep and assure that the chemical features of the pharmaceutical supplies are preserved.

Material and Methods

- **Plant material and extract preparation**

Calendula flowers were collected in the region of Campo Largo - PR in September 1998. The flowers were dried by the grower in a stove at 40º C/48h. A numbered voucher specimen was deposited at the herbarium at the Stave University in Ponta Grossa. The plant was submitted to preliminary quality assessment through ashes determination, humidity level and microscopic and macroscopic analysis with a magnifying glass in order to check the presence of contaminants and/or strange elements, at %.

- **Material distribution**

From the lot, 1350g were distributed in 45 plastic packets (polypropylene) with 30g capacity (weighed on an on-view-plate scales). The samples were directed to:

a) a stove: 9 packets (30g) were submitted to a temperature of 40º C (±2) for a 90-day period,

b) a refrigerator: 9 packets (30g) were submitted to a temperature of 5º C for a 90-day period,

c) a freezer: 9 packets (30g) were submitted to a temperature of -5º C for a 90-day period,

d) centrifuge: 9 packets (30g) were submitted to 3.000rpm for 60 minutes. This procedure was repeated 36 times, with the samples being distributed homogeneously for 90 days during the accelerated experiment,

e) the environment temperature: 9 packets (30g) were submitted to an environmental temperature for a 90-day period.

- **Extract preparation**

The freezer, refrigerator, centrifuge, stove and environment samples were put together after 90 days from the beginning of the experiment, once these used to represent the same lot from a single grower. A fluid extract was prepared from this material by hydro alcoholic 90% by percolation for ten days in a glass container and protected from the light. After this period the extract was filtered. The same procedure was adopted for the drug from the same lot which was submitted to the accelerated stability test. Parallel the production of the extract was accomplished with a sample from the same lot which did not undergo any ageing processes as a reference.

- **Assesment of the results**

Chromatografic method: the phytochemical profile of the extracts before and after the experiments were assessed by High Peroformance Liquid Chromatography (HPLC), in reverse phase.

- **Chromatography conditions**

stationay phase: RP18
mobile phase: solvent A (0.3%H3PO4 + 0.01M H2SO4) solvent B: ACN, solvent C: water
chrome type: 206 to 452 nm
injection:50µl

Results

- **Microscopic assessment**

Table 1 presents the results which were obtained in the macroscopic analysis expressed at % dirtiness, humidity level and ashes level which were found in the lot before the beginning of the stability test

	pharmacopoeia recommendation (at %)	findings in the analysed lot (at %)
ashes level	no more 9	8.3
insoluble ashes level	no more 2	0.005
dirtness level	no more 5	1.2
humidity level	no more 10	9.79

- **Comparative assessment of the chromatograms of the extracts:**

Figure 1 represents the phytochemical profile of the extract which was obtained from the flowers which were not submitted to the stability test;

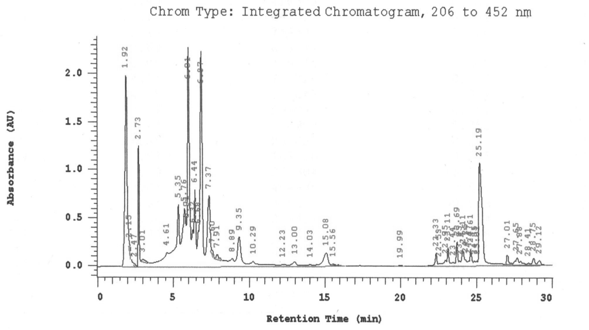
Figure 2 represents the phytochemical profile of the extract which was obtained from the flowers mixture when submitted to the stability test.

Conclusions

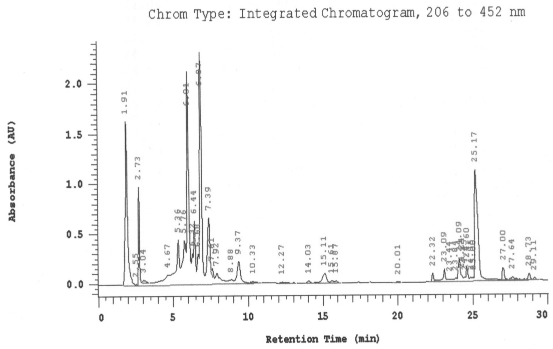
As for the preliminary tests the results were compatible with those prescribed by the consulted pharmacopoeia.

According to the results which were presented by the chromatograms it is observed that there were not any alterations to the phytochemical profile of the extracts. From this, it is concluded that the prolypropylene was proved effective in preserving the chemical characteristics of the vegetable drug. Its use for storage and marketing of the *Calendula officinalis* L. dried and stabilized is fully satisfactory.

This study is relevant due to the fact that this species is a available to the consumer in polypropylene packaging without any specific information about the storage conditions.



(figure 1)



(figure 2)

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QC-15 EVALUATION OF BIOAVAILABILITY OF SOLID PHARMACEUTICAL SPECIALITIES AT BASE OF TAMOXIFEN CITRATE MARKETED IN BRAZIL

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Introduction

Tamoxifen Citrate is available under tablet form, being largely employed as an endocrine therapy against breast cancer [3].

The objectives of our study was to evaluate the biodisponibility of Tamoxifen pharmaceutical formulation - Libra (Zita ®) - , comparing to the reference formulation - Tamoxifen (Wyeth-Whitehall®)-, all presented as tablet's form containing 10mg of the active drug .

Experimental methods

- **Formulations**

Formulation tests Tamoxifen; Libra (Zita®), and the reference formulation, Tamoxifen (Wyeth-Whitehall ®), all presented as tablet's form containing 10mg of active drug.

- **Animals**

To the study, we used ten (10) non-defined race dogs (RND) divided in two (2) groups .

- **Clinical Protocol**

Unless to ingest water *ad libidum*, the animals fasted for about 10 hours, at least, before the rehearsal, up to 6 hours, at least, after ingest medicine. After taking medicine, the animals received standardized ration and were free to drink water at will. The medicine's ingestion was supervised by the researcher and developed under cares of a veterinarian responsible by the kennel.

- **Clinical Parameters**

Pre-essays dose

- Complete clinical exam;
- Complete laboratories trace

Daily test

The animals were evaluated by the researcher and the veterinarian, in order to relate the possible collateral effects .

- **Experimental Project**

Randomization scheme

The medication was administered by a crossed rehearsal comprehending two stages. In the first, the animals were divided in two groups, in order to receive the medication according to following scheme (Tab. 1):

TABLE 1 - Study group

Animals	Period I	Period II
Group I	A	B
Group II	B	A

Dosage Administration

The animals in the first group (I) received four (4) units (10mg) of the only one dose preparation A, in the first day at 6:00 am. At the same time animals in group II received (4) units (10mg) of the only one dose preparation B.

After a 70 days leisure phase, the animals of the first group (I) received four (4) units (10mg) of the B preparation at 6:00 am. At the same time the animals of the second group (II) received four units (10mg) of A preparation. Both doses were administered as an only-one.

Sample Time

To evaluation of Tamoxifen's plasmatic concentration, were collected blood sample of 10mL by veined puncture at t_0 (basal - right before administration) and after the drug administration in the following times:0,5; 1; 2; 3; 4; 5; 6; 8;12;24;36 hours: in 7th, 14th and 21th days.

- **Extraction Sample in Plasma and Determination of Tamoxifen for the HPLC method with detection in U.V.**

The blood samples were collect in tubes containing EDTA and centrifuged, to separate the plasma. The plasma was transferred for sterile flasks and stocked at –70°C for subsequent analysis. Collected plasma samples of the animals that received medication, were spiked with 100 μL of a clomifen solution (internal standard) corresponding to 2 mg/mL. It was added 3mL of 1M hydroxide ammonium solution and 5mL ether. The mixture was shaken in vortex and centrifuged to 4°C for 20 minutes at 2000 rpm. The aqueous phase was frozen in bath of dry ice and acetone, and the ethyl phase was separated and evaporated up to dryness under flow of nitrogen and the residue was reconstituted with 1mL of methanol.

It was developed and validated the method of tamoxifen determination in plasma for HPLC with detection in U.V. operated at 280 nm. The employed move phase was composed of solution 0,014% of ammonium acetate in methanol and acetic acid (99:1), with flow of the move phase representing 0,3mL/minuto [4].

- **Statistical Analysis**

Non-Parametric and Parametric statistical method (90% confidence interval for the parameters AUC_{0-504}, $AUC_{0-\infty}$, C_{max}, T_{max}) were employed to test for bioequivalence (Table 2).

Results and discussion

In initial times there was not a good agreement between Zita and Tamoxifen (among 1-4 hours). Tamoxifen in the second hour (2;000) presented plasmatic concentration of 40,59 ng/mL, meanwhile Zita shown 16,75 ng/ml of that concentration, being totally eliminated after 24 hours in both cases (Figure 1).

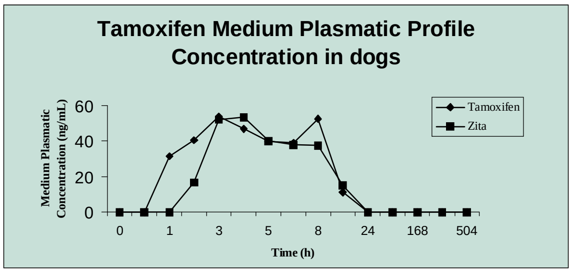

Figure 01 - Tamoxifen and Zita tablets medium plasmatic profile.

The medium bioavailability was calculated by the percentile rate Zita:Tamoxifen for the geometric average considering the values AUC_{0-504}, $AUC_{0-\infty}$, C_{max}, T_{max} , that resulted 86,18, 73,28, 81,41 e 76,29, respectively.

Table 2 – Non-Parametric statistical analysis of the values to 90% of Confidence Interval.

Bioavailability Rate (Zita vs Tamoxifen)				
Methods	AUC_{0-504}	$AUC_{0-\infty}$	Cmax	Tmax
Westlake's	67,1-132,8%	51,4-148,5%	54,9-145,0%	13,4-186,5%
Zita vs Tamoxifen				
Data linear	59,8-123,5%	49,8-149,7%	50,7-88,8%	87,0-257,2%
Data transformed	60,4-125,9%	53,8-153,0%	51,6-84,1%	14,6-185,3%
Period 2 vs Period 1	62,7-129,0%	38,2-120,8%	53,7-93,4%	46,9-133,2%
Two one-sided t Test	0,2791	0,1877	0,9095	0,6618

Conclusion

The values of AUC_{0-504}, $AUC_{0-\infty}$, Cmax, Tmax, for Zita and Tamoxifen formulations in plasma, situated out of the admitted strip (80-125%), and this did not attest, at all, a possible bioequivalence[2]. We accomplished the parametric calculus tests, which were not satisfied. Indeed, these results, represented values totally out of admitted strip when compared to parametric tests. The bioavailability data in plasma tamoxifen of the dogs, were not enough to indicate the bioinequivalence or not among the products Zita and Tamoxifen considering the reduced number of animals employed in the experiment and the large variability of tamoxifen plasmatic levels in dogs [1].

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QC-16 COMPATIBILITY AND STABILITY STUDIES OF ALPHA-METHYLDOPA AND MICROCEL™ BY THERMOGRAVIMETRY AND DIFFERENTIAL SCANNING CALORIMETRY

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Introduction and Objectives

As in a solid dosage form the drug is in an intimate contact with one or more excipients, frequently interactions may occur between these components with important changes in physical and physicochemical behavior and loss of stability. Even though Differential Scanning Calorimetry (DSC) cannot replace chemical methods for quantitative determinations of drug concentration in long term stability tests, it gives fast and adequate data to classify acceptable and unacceptable excipients, through the appearance, shift or disappearance of endothermic or exothermic peaks, and variations in the enthalpy values in DSC profiles of drug-excipient combinations by the comparison of their individual profiles. In sum, thermogravimetry (TG) gives important informations on the stability of solid dosage forms [1-3].

The aim of this work is to evaluate the behavior of the Alpha-methyldopa (**α-MD**), a widely used antihypertensive, microcristalline cellulose, Microcel™ 101 (**MC**), an excipient used in tablet formulations, and its physical mixture by TG and DSC analysis.

Experimental

α-MD was supplied by NUPLAM (Núcleo de Pesquisa em Alimentos e Medicamentos, UFRN), **Microcel MC 101 (**BLANVER FARMOQUÍMICA LTDA). The 1:1 binary mixture (**α-MDMC**) was prepared by grinding the specimen in a mortar. DSC curves were performed in a SHIMADZU – DSC 50 apparatus at 10°C heating rate under nitrogen flow of 50mL min^{-1}, from 30ºC up to 500°C. TG curves were performed in a SHIMADZU TG 50 – H apparatus, at 10°C, 15ºC and 20ºC heating rate under nitrogen flow of 50mL min^{-1}, from ambient up to 900°C for pure **α-MD** and **α-MDMC**. TG curves of pure **α-MD** and **α-MDMC** were analyzed with aid of TASYS software from SHIMADZU and the kinetic parameters A_e (activation energy) and Z (frequency factor) were obtained from the firs step of decomposition using the mathematical model of Ozawa.

Results and Discussion

TG curve of **α-MD** (Figure 1 and Table 1) showed four losses of mass. The first step is due to the loss of 1.5 moles of water and the next three steps correspond to the decomposition of the drug. DSC curve of **α-MD** showed two endothermic peaks (Figure 1 and Table 2). The first peak is due to the loss of crystallization water and the second peak correspond to the fusion. The superposition of the fusion peak between 306ºC and 325ºC (DSC) and the onset temperature (320º) of the first step of degradation (TG) shows that the fusion and decomposition of the drug occurs simultaneously.

TG curve of **MC** shows three losses of mass (Figure 1 and Table 1): the first with loss of 3.9% between 53ºC and 134ºC is due the dehydration; the second and third losses between 313°C and 362°C and 362°C and 516°C with losses of mass of 76.3% and 16.7% respectively are due the degradation reaction.

DSC curve of **MC** (Figure 1 and Table 2) showed two endothermic peaks, the first due to the loss of moisture water and the second due to degradation of the polymer. The degradation occurs from the dehydration and depolymerization of the polymer forming levoglucosan that degrades in CO, CO_2, H_2O and volatile combustible compounds [4-6].

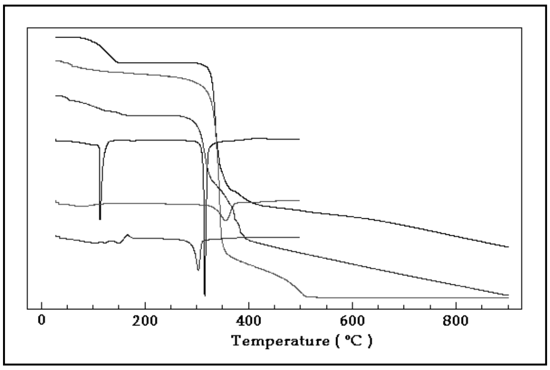

Figure 1: TG and DSC curves for **α-MD** (black)**, MC** (red) and **α-MDMC** (blue).

DSC curve for **α-MDMC** shows one endothermic peak between 282ºC and 309ºC instead of fusion peak of **α-MD** and the degradation peak of **MC**, with a intermediary ΔH value between the two specimens and shifting the onset temperature to lower value (282ºC). It was also observed a new exothermic peak at 160ºC. Those results indicate interaction between the two specimens.

Table 1: TG data of **α-MD** and **MC**

SPECIMEN	T_i/ºC	T_f/ºC	Δm/%
α-MD	96	135	10.4
	320	354	52.9
	374	404	6.3
	533	784	16.5
MC	43	75	4.0
	321	358	78.3
	432	513	15.1

Table 2: DSC data of α-MD, MC and α-MDMC (+:exothermic; **-**: exothermic)

SPECIMEN	T_i/ºC	T_f/ºC	PEAK/ºC	ΔH/Jg^{-1}
α-MD	104	129	114	-30.4
	306	325	315	-51.4
MC	50	132	76	-53.5
	324	380	356	-328.6
α-MDMC	46	119	103	-23.6
	119	136	124	-3.4
	136	159	150	-26.5
	160	175	167	+14.8
	282	309	304	-211.5

Concerning to the kinetics data, the onset temperature for the first step of decomposition **α-MDMC** is 30ºC below the onset temperature of **α-MD** (Table 3). The lower values of A_e and Z of **α-MDMC** points higher stability of pure drug when compared with the binary mixture probably due the effect of pyrolysis products of **MC** on the oxydation process of **α-MD.**

Table 3: kinetic parameters of thermal decomposition of **α-MD** and **α-MDMC.**

Sample	t_i/ºC	A_e/$kJmol^{-1}$	Z/min^{-1}
α-MD	320.0	152.15	1.012×10^{13}
α-MDMC	290.0	115.27	9.029×10^{9}

Conclusions

The analysis of the results shows that the alpha-methyldopa interacts with Microcel™ reducing the stability of the drug. So **MC** can not be used as excipient in pharmaceutical solid dosage forms containing **α-MD**.

Acknowledgement

The authors thank to CAPES for the financial support.

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QC-17 SHELF LIFE ESTIMATIVE OF HYPOGLYCEMIC TABLETS FROM ISOTHERMAL THERMOGRAVIMETRIC DATA

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Introduction

The stability with time under given climatic conditions is a very important property of a new pharmaceutical product. Stability studies are routinely conducted by the pharmaceutical industry in order to evaluate any kind of chemical and physical degradation of the product [1-3]. Individual samples are taken at predetermined time and analysed in order to verify the decrease on the active drug content, increase on the degradation product content and dissolution behaviour changes. The specifications of new pharmaceuticals depend of these results. This means that the stability data are very important for determination of the most of the main quality characteristics, such as efficacy, safety and technical feasibility [4-5].

There are several methods which may be used to estimate stability characteristics, including isothermal method and determining reaction kinetics from either a single scan or from a number of scans derived at different heating rates[5].

The present work proposes to evaluate the stability data of the hypoglycemic drug and its tablets by thermal analysis.

Materials and methods

- **Samples**

The excipients starch, explocel, lactose, talc, magnesium stearate and glibenclamide drug and tablet A were obtained in the Laboratory of Pharmaceutical Technology of the Federal University of Paraíba (LTF-UFPB). Glibenclamide and chlorpropamida tablets B was acquired in local drugstore.

- **Thermogravimetric**

Glibenclamide tablet test was elaborated with the following composition: glibenclamide 5.3%, starch 51.5%, lactose 37.1%, explocel 2.8%, talc 2.1% and magnesium stearate 1.2%. Chlorpropamide tablet test was elaborated with the following composition: chlorpropamide 49.50%, starch 39.66%, PVPK30 4.95%, explocel 2.80%, talc 2.10% and magnesium stearate 0.99%.

TG isothermal curves of the glibenclamide and chlopropamide drug and tablets reference and test were obtained in a Shimadzu thermobalance model TGA-50H. under flow of air of 10mL/min., during 4 hours, at 190, 180, 170, 160 and 150^{0}C for glibenclamide and 170, 160, 150, 140, 130 and 120^{o}C for chlorpropamide.

The rate constants were calculated by Arrhenius equations and its reaction order was determined.

$$k = A\exp\left(\frac{-E}{RT}\right) \quad (1)$$

where k is a specific rate constant at temperature T (K) , A is the Arrhenius frequency or pre-exponential factor, E is the activation energy and R is the gas constant. It is generally assumed that k is derived from first-order kinetics and at isothermal temperatures. However many solids decay by non-first-order reactions and the thermal data need careful mathematical manipulation before accurate and reliable interpretation may be made.

- **Results and discussion**

A procedure for statistical evaluation of stability data for the purpose of shelf life determination–not for the stipulation of the specifications for the quality characteristics itself-is described in the U .S. Food and Drug Administration (FDA) guidelines (1). It consists of the calculation of individual shelf life of the products.

The dynamical TG curves of the glibenclamide drug presented three stages of thermal decomposition reaction and tablets A and B presented four stages each one. TG dynamic curves of the chlorpropamide drug presented one stage , tablets A presented three stages and B presented four stages of thermal decomposition,evidencing similar thermogravimetric profiles in the first stage. From the second and third stages compared to the tablets A and B occurred mainful differences between A and B in relation to the mass loss. The third decomposition was similar between our. Only tablet B presented fourth step of thermal decomposition reaction.

The isothermal thermogravimetric data was used to determine the reaction order and rate constants of thermal decomposition reaction The values of the rate constants and shelf life, Table I, showed that the formulations A and B presented more stability than glibencamide drug. The formulation B is more thermally stable than formulation A.

The results of chlorpropamide are presented in Table I, where can be verified a major rate constant value and shelf life for the chlorpropamide drug in all temperatures compared to the tablets A and B.

Of course, safety considerations have the highest priority in the list of factors that affect the setting of specifications. However, fortunately the quality standards in pharmaceutical industry are so high that usually the quality of a drug product is of no safety concern, and thus specification limits are usually not based on safety considerations. Rather, in most cases the observed quality of all ragistration stability products throughout their life forms the basis for specification setting.

The expiration dating period (shelf life) showed that the isothermal TG data showed that is possible to determine the stability of similar products differentiating them (table-I) .

Table 1. Kinetic parameters of decomposition of the hypoglycemic drug and its tablets A and B.

sample	Ea (kcal.mol^{-1})	k_{25^oC} (s^{-1})	Shelf Life (Years)
Glibenclamide	19.23	1.10×10^{-9}	3.04
Tablet A	21.97	1.74×10^{-11}	3.20
Tablet B	21.63	1.33×10^{-11}	4.19
Chlorpropamide	19.90	1.12×10^{-9}	2.97
Tablet A	20.07	8.33×10^{-10}	4.01
Tablet B	20.26	5.66×10^{-10}	5.91

Conclusion

The rate constants of thermal decomposition reaction and shelf life reveal differences between tablets A and B each hypoglycemic drugs, showing to treat of products with different formulations.

Acknowledgements

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QC-18 THERMAL STABILITY OF ACETAMINOPHEN DRUG AND TABLETS

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Introduction

The acetaminophen is a active metabolite of phenacetin, an analgesic derivate of tar. It is an alternative effective like analgesic and antipyretic, but its anti-inflammatory action is weak.

The thermal analytical techniques have been used to characterize the behavior of the sample as function of temperature. These methods include differential scanning calorimetry, DSC, differential thermal analysis, DTA, and thermogravimetry, TG. Nowadays these techniques constitute method of choice at thermal characterization of drug and its formulation [2].

A pre-formulation study is a phase in the development process in which the physical, chemical and mechanical properties of a drug are characterized, in order to develop stable, safe and effective dosage form [1,2].

In the preset work, TG, DTA and DSC coupled to the photovisual system were used to determine the thermal parameters of acetaminophen drug and tablets in a comparison of different formulations.

Experimental Methods

- **Materials**

The acetaminophen drug and pharmaceutical excipients (aerosil 200, avicel 101 and 102, croscarmellose sodium, dibasic calcium phosphate, lactose, magnesium stearate, povidone, sodium starch glycolate, starch and talc) were donated by LTF/UFPB. The tablets 750 mg A and B (standard) were acquired from a local drugstore.

The binary mixtures were manufactured in the LTF/UFPB, with the following composition (acetaminophen:excipient): 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10.

- **Techniques**

The non-isothermal and isothermal TG curves were obtained with a Shimadzu model TGA-50H thermobalance under an atmosphere of air at flow rate of 20 mL/min., at a heating rate of 10 ºC/min. up to 900 ºC. The sample mass was about 8 mg. The isothermal TG curves were measured at 190, 200, 210, 220 and 230 ºC during 240 minutes. The rate constants of the thermal decomposition reaction were determined from the TG data using the Arrhenius expressions.

The DTA curves were obtained with a Shimadzu model DTA-50 under an atmosphere of nitrogen at flow rate of 50 mL/min., at a heating rate of 10 ºC/min. up to 500 ºC.

The DSC curves were obtained with a Shimadzu calorimeter model DSC-50 coupled to a photovisual system, in the temperature range 25 – 500 ºC, under an atmosphere of nitrogen at a flow rate of 50 mL / min. The samples were analysed at a heating rate of 10 ºC/min. up to 500 ºC.

Results and Discussion

The TG curves showed that the drug and tablets presented three and four decomposition processes, respectively. The samples too presented the same thermal behavior, but at temperature about 300 ºC was observed the finish thermal d e mineral r

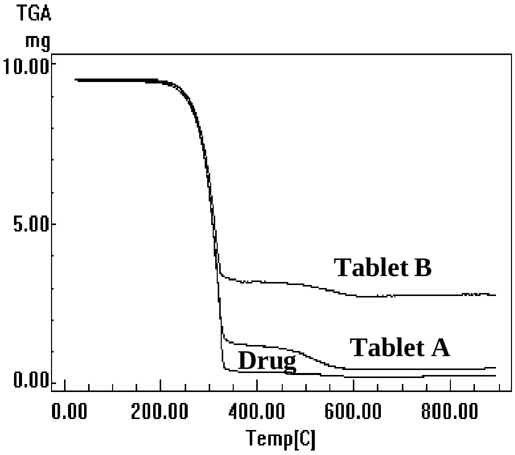

Figure 1 – The TG curves of drug and tablets of acetaminophen.

The rate constants are in agreement with the classical Arrhenius kinetics and revealed that the drug and tablets of acetaminophen to present the same stability (Table 01).

The study of compatibility drug – excipients by DTA showed that all the excipients, except povidone, interact with acetaminophen, increasing its stability.

Table 1 – Rate constants of thermal decomposition reaction of acetaminophen drug and tablets.

Temperature (^oC)	**Rate Constant (k. s^{-1})**		
	Paracetamol		
	Drug	Tablet A	Tablet B
190	9.63×10^{-6}	1.01×10^{-5}	8.25×10^{-6}
200	1.54×10^{-5}	1.48×10^{-5}	1.84×10^{-5}
210	2.98×10^{-5}	2.20×10^{-5}	3.33×10^{-5}
220	4.47×10^{-5}	4.62×10^{-5}	3.96×10^{-5}
230	8.76×10^{-5}	8.18×10^{-5}	7.96×10^{-5}

The DSC curves of acetaminophen drug, tablet A and B showed an endothermic peak corresponding to the melting point at 170.2, 169.5 and 169.2 ºC, respectively (Figure 02) according to literature [3] that is of 169.0 – 170.5 ºC.

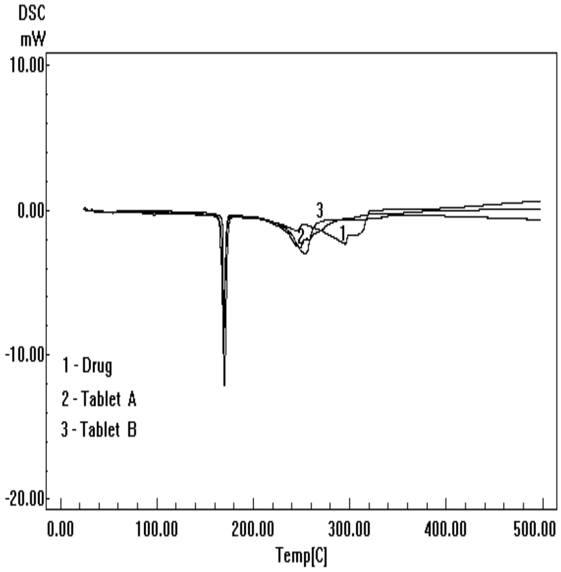

Figure 2 – DSC curves of acetaminophen drug and tablets.

DSC photovisual presented in the Figure 3 show the pictures of acetaminophen drug and tablets. The melting process is accompanied by a mass loss in TG curve which reveal that the phase transition is solid ⇒ liquid + gas. The pictures A, B and C (Figure 03) still reveal different colors in the decomposition processes of tablets A and B. Such fact can indicate different chemical compositions in the final product. The DSC photovisual studies allowed to visualize small differences in thermal behavior between the two tablets. The DSC and TG curves show similarity between two formulations.

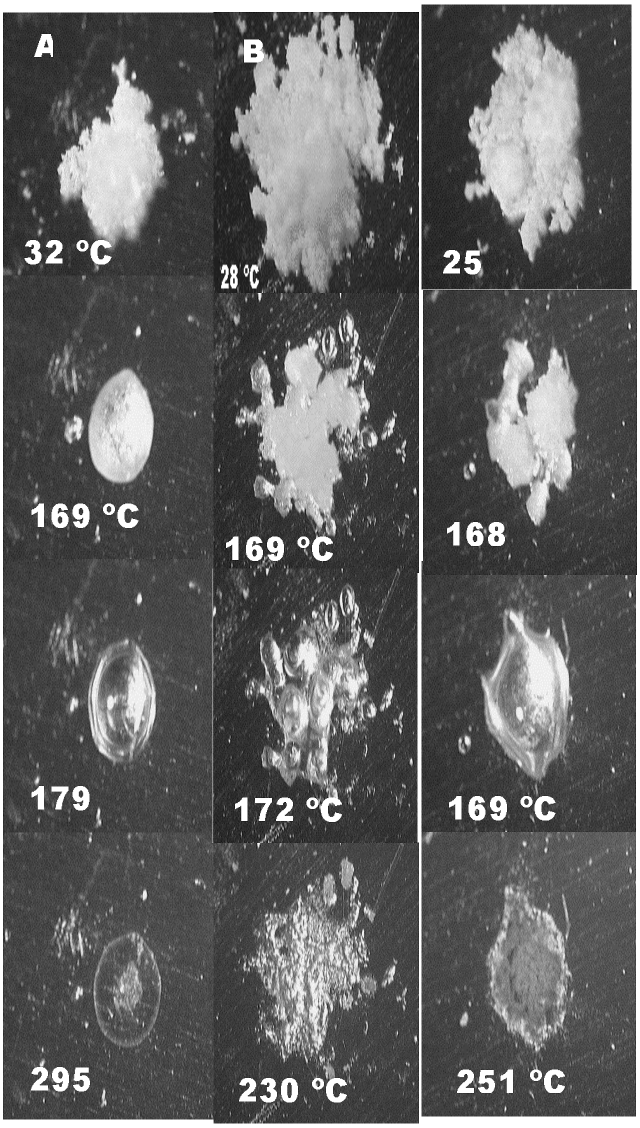

Figure 3 – DSC photovisual of acetaminophen drug (A), tablet A (B) and tablet B (C).

Conclusion

The kinetic and DSC photovisual studies allowed an assessment of the pharmaceutical equivalence of two acetaminophen formulations.

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QC-19 EVALUATION OF MANIOC INDUSTRIAL PROCESSING RESIDUE AS A DISINTEGRANT FOR COMPRESSED PRAZIQUANTEL TABLETS

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Introduction

Starch is a naturally occurring substance of wide distribution and a major adjuvant in the formulation and production of tablets. Starches derived from wheat, rice, corn (maize) and potato have been accepted by the British Pharmacopoeia and are used in tableting as diluents, binders, disintegrants and lubricants.

Farelo is a residue from the industrial process of *Manihot esculenta* in Brasil. It contains a high concentration of starch and it has been discharged into rivers with damaging effects to the environment. There is no information using *farelo* as a tablet disintegrant reported in the literature. In recent study (data unpublished) we have compared the disintegration efficiency of *farelo* with cornstarch in placebo tablets. We found that *farelo* present better disintegrant power and less binding ability than cornstarch.

The aim of the present study has been to investigate the disintegrant efficiency of residue of manioc from industrial processing (*farelo*) in tablets containing praziquantel (PRZ) as a drug model. The physical characteristics of these tablets have been compared with those using cornstarch. The confirmation of its disintegration properties may offer an alternative disintegrant of quality at low cost.

Experimental methods

- **Materials.** Praziquantel, lactose, cornstarch, *farelo*, PVP (10% alcoholic solution), magnesium stearate, talc.

Preparation of tablet formulation and granules

The tablet formulations are given in Table 1. For each formulation batch (300g), the drug and the disintegrants, either *farelo* or cornstarch were weighed and dry-mixed by the geometric dilution method employing a porcelain mortar and pestle. The dry mixture was then manually mixtured in a plastic bag for five minutes. An apropriate quantity of freshly prepared solution of PVP (10% w/v) was then added as the granulating agent and mixed until a uniform wet mass was obtained. The wet mass was then granulated by passing through a sieve. The granules were dried at 40°C until 2% moisture and the resulting dry granules were passed through a 840 µm sieve.

- **Size distributions of the granules**

The particle size distributions of the granules were measured by sieve analysis.

Table 1: Formulations of PRZ tablets (mg).

	F10P	F20P	S10P	S20P
PRZ	200	200	200	200
lactose	360	320	360	320
farelo	40	80	-	-
cornstarch	-	-	40	80

- **Compression of tablets**

The granules which passed a 177 µm sieve were rejected. 1% magnesium stearate and 1% talc were mixed immediately before compression. The granules were compressed in a 12 mm diameter stainless steel die fitted with plan punches by a single punch tablet machine (Korsh™ EKO, Germany).

Measurement of tablet properties

- **Weight Uniformity**

Fifty tablets from each batch were weighed in an analytical balance (Ohaus™ AS 200) and the mean value and the standard deviation were calculated.

- **Thickness**

The thickness of ten tablets (in triplicate) from each batch was measured on a Mitutoyo™ digital micrometer (precision 0.001mm).

- **Crushing strength**

The *Schleuniger Pharmatron*™ (6D) tablet hardness tester was employed for establishing the crushing strength of the tablets. Fifty tablets from each batch were used in each determination and the mean value and the standard deviation were calculated.

- **Disintegration time**

The disintegration time of 18 individual tablets was determined in distilled water and in a laurilsulfate sodium solution (2mg/ml) at 37°C using the USP XXIII procedure for determination in an Etica™ apparatus.

Results and discussion

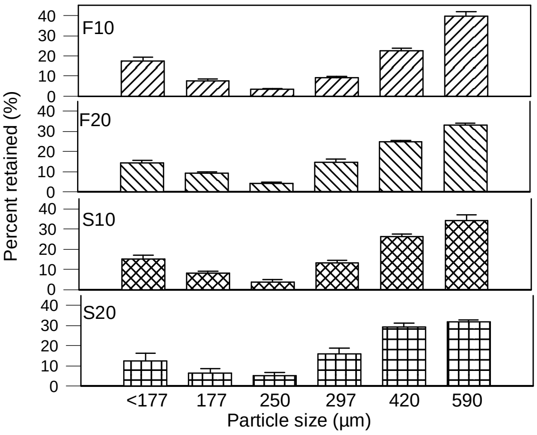

Figure 1: Particle size distribution of the granules.

The results were calculated by the percentage of each granulometric group. The size distributions of the granules prepared from *farelo* and cornstarch showed similar profile.

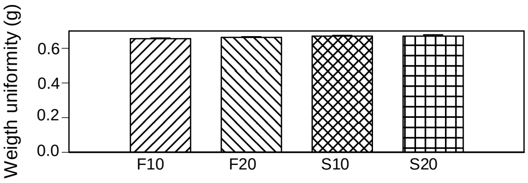

Figure 2: Weigth variation of tablets.

The results of the uniformity of weight of the tablets are showed in Figure 2. Examination of the data indicated that all tablets passed the USP test for weight uniformity. All batches showed a minimum variation in the weights of their tablets. These results were reflected by the minimum values of their standard deviations of weight uniformity (error bars).

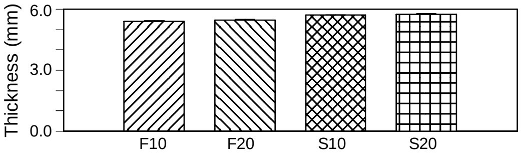

Figure 3: Thickness of the tablets.

All the batches exhibited a minimum variation on thickness. Such results proved that changing the type or concentration of disintegrant did not affect the flow properties of granules, since, it was reported that the changes in tablet thickness manifested a problem in the flow properties of granules.

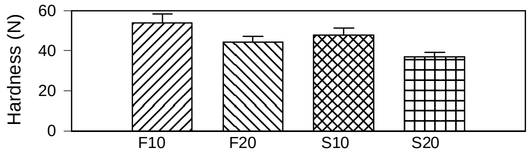

Figure 4: Crushing strength of the tablets.

There is no significant difference between each batch. However tablet with *farelo* showed higher values of hardness. The analysis of these data together with those of weight variation allow us to state that PRZ granulate with corntarch results in greater difficulty in manufacturing tablets than PRZ with *farelo* since there has been the need of expanding the die and increase compression force.

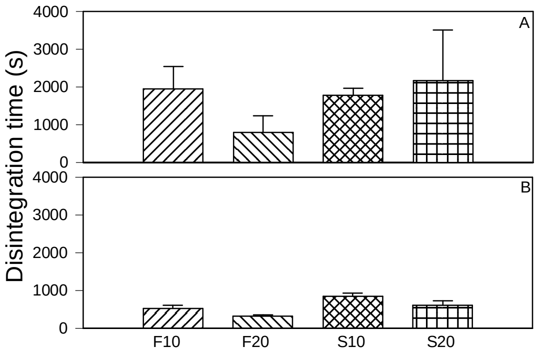

Figure 5: Disintegration time of the tablets. Medium: A: water, B: laurilsulfate sodium solution (2mg/ml).

The disintegration time of the tablets is of critical importance with regard to their efficacy. Figure 5 compares the disintegration times carried out under different media, either water or laurilsulfate sodium solution. As expected the disintegration time of the tablets in laurilsulfate sodium solution was shorter than in distilled water at 37°C. Figure 5 showes that disintegration time of PRZ tablets is different according to the disintegrant used and its concentration. The tablets containing *farelo* disintegrated faster than those contaning corntarch.

Conclusion

The granules have shown similar size distribution. All the tablets have shown low weight and thickness variations and appropriate hardness. The disintegration time was different according to the disintegrant. Tablets with f*arelo* exhibited the best disintegration time. *Farelo* exhibited good performance as a disintegrant agent for compressed tablets in the studied conditions.

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QC-20 QUALITY EVALUATION OF ANALGESIC AND ANTITHERMIC PHARMACEUTICAL PREPARATIONS USED IN THE PAEDIATRIC HOSPITAL OF NATAL/BRAZIL

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Introducion

In the class of analgesics and antipyretics pharmaceutical preparations produced in Brazil, the acetylsalicylic acid (ASA), paracetamol and dipyrone are the most often used. The ASA is a pharmaceutical formulation used as tablets, while the paracetamol and dipyrone are used in paediatrics in the form of liquid preparations.

Objectives

The present work has the objective of evaluating the quality of the pharmaceuticals preparations of ASA, paracetamol and dipyrone of paediatric used in Paediatric Hospital of Natal/Brazil as well as stabilishing comparasions between price of acquisition and product quality.

Materials

The samples comprised eight lots of ASA tablets of 100mg, fifteen lots of paracetamol 20% solution and twelve lots of dipyrone 50%, produced for diferent pharmaceutics firms and used in the Paediatric Hospital of Natal/Brazil.

The analyzed samples were represented in this study by alphabetics letters.

The pharmaceuticals formulations were packed in plastic containers of 10mL and 15mL in the case of the dipyrone and paracetamol. The ASA tablets were packed in plastic, paper and aluminium strips. All samples were into the expiration date.

Methods

In the quantitative determination of the ASA the method of the neutralization titrations was used, in agreement with the British Pharmacopeia from 1993[1]. For the determination of the paracetamol the ultravioleta spectrofotometry at 275nm was used, again in agreement with the British Phamacopeia. In the case of the dipyrone the method used was that of oxidation-redution-iodimetric, now in agreement with the German Phamacopeia [4].

In all samples of the ASA, paracetamol and dipyrone identification tests were made.

The control of phisical properties in phamaceuticals forms was done throught analysis of weight variation, desintegration rate, hardness, friability [3] and dissolution test [10] to the ASA tablets and the throught weight variation, cleanness and pH to dipyrone and the paracetamol oral solution.

Results and Discussion

Figure 1 shows the results of the quantitative determination respect to ASA, dipyrone and paracetamol pharmaceutical formulations.

The results of the control of physical properties of the ASA tablets are presented in Tabel 1.

The Table 2 shows the results of physicals properties of paracetamol and dipyrone oral solution.

Table 1: Physical Properties in Pharmaceutical Forms - ASA tablets.

SAMPLE	FRIABILITY (<15%)	WEIGHT VARIATION (mg)	HARDNESS (Kgf/cm^2)	DESINTEGRATION RATE (15')	DISSOLUTION TEST
A	1,09	0,1475	4,92	1,00	100
B	0,135	0,1499	5,95	1,00	97,7
C	0,87	0,153	3,00	1,00	61,64
D	0,6	0,1225	4,55	1,00	100
E	0,48	0,1472	5,60	1,00	88,09
F	0,48	0,1209	4,80	2,40	100
G	0,34	0,1572	5,85	1,00	92,87
H	0,55	0,1707	3,74	1,00	100

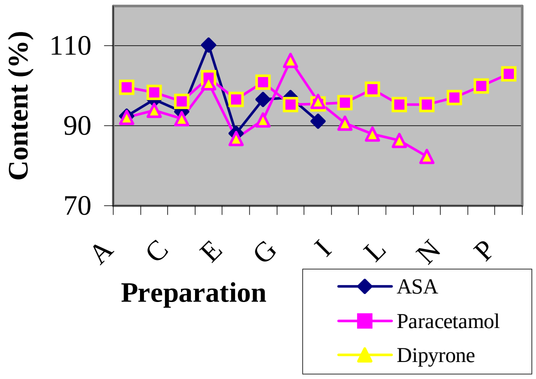

Figure 1: Results of the quantitative determination of ASA, Dipyrone and Paracetamol in Pharmaceutical Forms.

Table 2:Physical properties in pharmaceutical forms - Paracetamol and Dipyrone oral solution

Samples		**Weight variation**		**pH**	
Paracetamol	**Dipyrone**	**Paraceta Mol**	**Dipyrone**	**Paracetamol**	**Dipyrone**
Ap	**Ad**	15.0	9.6	5.3	6.5
Bp	**Bd**	15.0	9.6	6.0	6.5
Cp	**Cd**	7.5	10.0	5.9	6.0
Dp	**Dd**	15.0	10.2	5.4	6.0
Ep	**Ed**	10.0	10.0	5.0	6.0
Fp	**Fd**	15.0	9.9	6.0	6.0
Gp	**Gd**	10.0	10.0	5.3	6.0
Hp	**Hd**	15.0	10.0	5.9	5.5
Ip	**Id**	15.3	9.9	5.2	5.5
Jp	**Jd**	10.0	10.0	5.6	6.5
Lp	**Ld**	15.3	10.0	6.1	6.0
Mp	**Md**	15.0	10.0	6.1	6.5
Np	**Nd**	15.0	10.0	6.0	6.0
Op	**Od**	15.0	9.9	5.8	6.0
Pp	**Pd**	15.0	9.6	5.3	6.5

Conclusion

After the analyses it was verified that only 12.5% of the specimens of ASA, 38.46% of the specimens of paracetamol and 8.3% of the specimens of dipyrone were in agreement with the quality specifications established by pharmaceuticals codes.

Further more, it was verified that price variation didn’t influence in the product quality.

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QC-21 MICROBIOLOGICAL ASSAY FOR ENROFLOXACIN

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Introduction

Enrofloxacin, (Fig. 1) a fluorquinolone marketed specifically for use in veterinary medicine, has been shown to be rapidly bactericidal against a broad spectrum of aerobic and facultative anaerobic bacterial including strains resistant to many other antimicrobial agents [1,2]. Enrofloxacin also has been reported to have activity against some obligate anaerobic bacteria. As with the other fluorquinolones, enrofloxacin inhibits DNA synthesis and is rapidly bactericidal against susceptible proliferating and dormant bacteria [3,4]. Chemically enrofloxacin is a 1 cyclopropil-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid [5] and is not official in any pharmacopoeia. The microbiological assay for the determination of enrofloxacin in pharmaceutical formulations has not been reported yet. The objective of the present study was to find a sensitive and reproducible agar diffusion method to quantify enrofloxacin in raw material and injectable solutions. This method can be applied to stability studies.

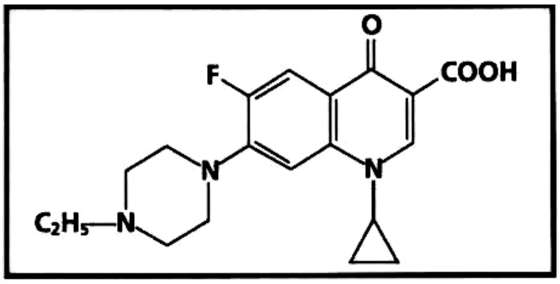

Figure 1. The chemical structure of enrofloxacin

2. Materials and methods

The enrofloxacin reference substance (assigned purity 99.28%) were generous gifts from Hoechst Vet. The enrofloxacin 5% injectable solution were obtained commercially. Analytical reagents grade chemicals were used.

- **Organism and inoculum**

The cultures of *Staphylococcus aureus* ATCC 6538p were cultivated on Antibiotic Medium 1 (Difco) at 35ºC for 1 day. Diluted cultures suspensions of 25 % turbidity were obtained at 580 nm, using a suitable spectrophotometer. Portions of 1,0 ml of the inoculated saline solution were added to 100 ml of Antibiotic Medium 1 at 48 ºC used as inoculated layer.

- **Enrofloxacin reference standard**

The standard solution was diluted in 0,1M potassium phosphate buffer, pH 8.0 and assayed at concentrations of 3.2, 6.4 and 12.8 µg ml^{-1}.

- **Assay of injectable enrofloxacin**

A quantify of the injectable enrofloxacin solution containing 500 mg (10 ml) de enrofloxacin were transferred volumetrically into 50.0 ml volumetric flasks, followed by making up to volume with 0.1M potassium phosphate buffer, pH 8,0, to give a final concentrations of 3.2; 6.4 and 12.8 µg ml^{-1}. All determinations were conducted in triplicate.

- **Cylinder-plate assay**

The agar was composed of two separate layers. The Antibiotic Medium 1 (21 ml) was poured into 100 x 20 mm Petri dish for the base layer. After solidification this layers portions of 4 ml of inoculated Antibiotic Medium 1agar was poured to the base layer. Six stainless steel cylinders of uniform size (8 x 6 x 10 mm) were placed on the surface of inoculated medium. Three alternated cylinders were filled with 100 µl of reference concentrations solutions on the other three cylinders with the concentrations samples solutions. Six plates were performed for each sample. After incubation (35°C for 18 h) the zone diameters (in mm) of the growth inhibition were measured using a caliper.

- **Calculation**

To calculate the activity of enrofloxacin the Hewitt equation was used. The assays were statistically calculated by the linear parallel model and by means of regression analysis and verified using analysis of variance [6,7,8,9].

- **Method validation**

The method was validated by determination of linearity, precision and accuracy.

Results and discussion

The activity of antimicrobial agents may be demonstrated under suitable conditions by their inhibitory effect on microorganisms [9].

The activity of enrofloxacin *in vitro* has been tested against various microorganisms. These studies have shown that enrofloxacin has potent *in vitro* activity against *Staphylococcus aureus* [2]. Although the microbiological assay methods involve many steps and are time consuming they can reveal subtle changes not demonstrable by chemical methods [9]. There are no official microbiological cylinder-plate assays described in the official codes to determine enrofloxacin in pharmaceutical formulations. In this experimental 3 x 3 design using three dose levels for each standard and sample were used following the procedure described in British (1993), European (1997), and Brazilian (1988) Pharmacopoeias.

The calculation procedures normally assume a direct relation ship between the observed zone diameter and logarithm of applied dose. The corresponding mean zone diameters for standard solution were: 15.98 ± 0.116 (RSD = 1.7) for low dose; 18.84 ± 0.023 (RSD = 0.30) for medium dose and 21.64 ± 0.023 (RSD = 0.26) for high dose. Corresponding zone diameters ranged between 15.78 – 16.85. 18.78 – 18.91 and 21.58 – 21.73 for concentrations of 3.2. 6.4 and 12.8 $\mu g\ ml^{-1}$. respectively. The calibration curves for enrofloxacin were constructed by plotting zone diameter (mm) versus log of concentration ($\mu g\ ml^{-1}$) and showed good linearity on the 3.2 to 12.8 $\mu g\ ml^{-1}$. The representative linear equation for enrofloxacin was $y = 11.24178 + 9.40043\ x$ ($n = 3$, $r = 0.99998$, $r^2 = 0.99996$). According British, European and Brazilian Pharmacopoeias, if a parallel-line model is chosen, the two log dose-response lines of the preparation to be examined and the reference preparation must be parallel and they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a given probability, usually $P= 0.05$. The assays were validated by means of the analysis of variance as described in these official codes. There are no deviation from parallelism and linearity with results obtained here ($P< 0.05$). The precision and accuracy of the assay were demonstrated. The repeatability shows mean D.P.R. 0.26% indicating good intra-day precision of the method. The mean recovery were found to be 99.23 %.

Conclusions

Therefore, the microbiological assay is satisfactory for quantitation of *in vitro* antibacterial activity of enrofloxacin.

The results indicate that this method is an acceptable alternative method for the routine quality control of enrofloxacin.

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QC-22 ANTIBACTERIAL ACTIVITY OF THIOSEMICARBAZONES FROM SUBSTITUTED PIPERONYLIDENACETALDEHYDE COMPOUNDS AGAINST *Staphylococcus aureus* STRAINS

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Introduction

In the latest years a dramatic increase in incidence of multidrugs resistance strains had been observed, due to mainly of minuse of antibiotics.

The *Staphylococcus aureus*, a prevalent nosocomial pathogen, presents serious problems since there are innumerous methicillin-resistance strains (MRSA) multidrugs resistant to several antibiotics of the same or different family. Some strains were sensitive only to the glycopeptides, vancomycin or teicoplamin. Nevertheless, some strains of coagulase negative Staphylococcus become resistant to vancomycin. (Schwalbe 1987) and also some *Staphylococcus aureus* had their sensitivity to vancomycin decreased (Hiramatser, 1997).

In this context, there are interest to search to new synthesis compounds with antibacterial activity potential.

Within the thiosemicarbazones group, interesting biological activities were observed, such as antibacterial (Dobek 1980) and antimalaria (Klayman, 1984). Meanwhile, relation structure – activity studies pointed that usually a chain is essential to biological activity. Basically the molecular modification in these compounds are made in the cyclical portion preserving the lateral chain of thiosemicarbazone. In this work were studied the in vitro antibacterial activity of two thiosemicarbazones piperonylidenacetaldehyde against sixteen *Staphylococcus aureus* strains.

Experimental methods

- **Materials**

Two new thiosemicarbazone derivatives of substituted piperonylidenacetaldehide , or 3–(3,4 methylenedioxy–phenyl)–2 propenal, have been prepared by condensation of the respective aldehydic derivative with substituted thiosemicarbazides in acetic acid. The new compounds have been characterized by infrared (IR) and proton nuclear magnetic resonance spectroscopy.

The compounds studied have the general structure

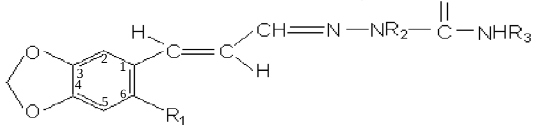

Table 1. Thiosemicarbazones Piperonylidenacetaldehyde

	R1	R2	R3	Molecular weight	Melting point	Sol. in DMSO
4g	NO_2	H	CH_3	308.32	236.5–238.5C°	Soluble
4h	NO_2	H	C_2H_5	322.34	227.7–229.0C°	Soluble

4g=4”-methyl-3”thiosemicarbazone3(3’4’methyl enedioxy- 6’nitrophenyl) 2 propenal

4h=4”ethyl-3”thiosemicarbazone3(3’-4’methyl enedioxy-6’nitrophenyl) 2 propenal.

- **Determination of *in vitro* antibacterial activity**

The antibacterial activity of the compounds was determined by the agar diffusion method, using the multi-inoculator of Steers.

The thiosemicarbazone of piperonylidena cetaldehyde (4g or 4h) mother solution are prepared in (DMSO) and followed by dilution. Finally the compounds are incorporated in Petri dish containing Mueller Hinton agar, with the concentration from 10 to 200 µg/ml.

The compound were tested against sixteen *Staphylococcus aureus* strains, of which, 4 were reference microorganisms (ATCC) and 12 clinically isolated. The reference strains are used in challengue test for preservative agents, meanwhile the isolated ones, eight were methycillin resistant and four were methycillin sensitive.

The inoculum was prepared in Müeller Hinton broth by adjustment of a log phase broth culture to a McFarland 0.5 turbidity standard. Sixteen. Staphylococcus strains were tested inoculating them on the surface of the plaque with Müeller Hinton agar, containing the different concentration of the thiosemicabazone, with one Steers replicator. We use tetracycline as antibiotic standard.

Results and discussion

The results obtained are presented in figure 1 with the phenotypes of the *Staphylococcus aureus* isolated against 24 antibiotics. There of the studied strains, the isolated *Staphylococcus aureus* AM 264, AM 270 and AM 283, are strains multiresistants (table 2) graphyc 1, only sensitive to Vancomycin.

At the determination of MIC of the thisemicarbazone of the 6 nitropiperonylideancetaldehyde (4h), 10 strains of Staphylococcus aureus presented MIC over 100µg/ml, 4 strains MIC of 100µg/ml and 2 strains MIC of 20µg/ml.

The thiosemicarbazone of 6 nitropiperonylideancetaldehide (4g) presented 2 strains with MIC of 10µg/ml, one *Staphyococcus aureus* Methicillin sensitive (MSSA) and one Methicylin resistant (MRSA) also resistant to Tetracycline.

As to the tetracycline used as standard antibiotic there were 8 strains resistant to it, (MIC, over 10µg/ml) and 8 sensitive strains with MIC less than 2µg/ml.

Table 2- Staphylococcus Multiresistance

Strains of S. aureus	**Antibiotics**					
	β-Lactam antibiotic	Amino glycoside	Quino lones	Macro lides	Tetra cycline	Chloran Phenicol
AM 264	09*	02	01	03	01	01
AM 270	08	02	01	03	01	01
AM 283	09	01	01	03	01	01

* Number of antibiotics tested in each family

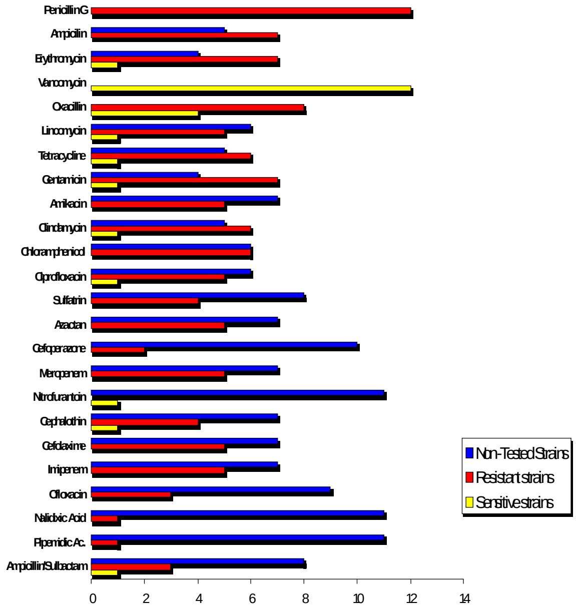

Figure 1. Phenotypes of isolates *Staphylococcus aureus*

Conclusion

From the studied compounds, the thiosemicarbazone (4g) presented a good activity, the same order of the tetracyline on the *S. aureus* AM 318 strain resistant to β-Lactam antibiotics, Penicillin G, Ampicillin, Cefazolin, as Quinolones, Macrolides and Gentamicin.

The same compound presented in previous against studies of screening, the antibacterial activity against the *Bacillus subtilis*, *Mycobacterium smegmatis*, *Candida albicans* and *Monilia sitophyla* although no evidence on Staphylococcus aureus

The thiosemicarbazone (4h) presented an interesting activity on two of the multiresistant strain: *Staphylococcus aureus* AM 270 and AM 283, with a MIC of 20µg/ml and 100µg/ml respectively.

It is considered that the presence of the nitro group on the position 6, increase in both cases the biological activity.

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QC-23 MICROBIAL QUALITY CONTROL OF COMMERCIAL SAMPLES OF *Tabebuia avellanedeae* Lor. ex Griseb. COMMERCIALIZED IN MARKETS OF SÃO LUÍS/MARANHÃO

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Introduction

The quality of the plants and/or preparations of origin vegetable maids as therapeutic resource suffers influence of several extrinsic and intrinsic factors, demanding the obedience ofthe ideal conditions of culture, crop, secagem, stabilization, manufactures, conservation and storage. In conservation and storage is indispensable the attendance the relative determinations to the atmosphere, division state, packing and period of validity, seeking to guarantee the quality of the vegetable material represented by the maintenance of the characteristics of authenticity, integrity and purity [1,2] .One of most frequent problems in the quality meets related the inadequate conditions of storage during the commercialization, exposing the vegetable material the dust, heat, humidity, rodents, microorganisms and other deleterious agents [1,2,3]. The contamination for bacterias and funguscan take the destruction and/or alteration of the active beginnings of the vegetable species, as well as to cause the appering of toxicant substances, besides neurotoxics, nefrotoxics, hepatotoxics and cancerous; exposing, like this, the user to serious damages the health or the own life [1,4,5].

In the State of Maranhão (Brazil), the use of plants and/or preparations of vegetable origin as therapeutic resource is a practice that comes passing of generation the generation, being verified that most of the population from Maranhão, when acquiring such products, it falls back upon the trade retailer accomplished in markets and free markets, where they are precarious the hygienic-sanitary, inadequate conditions the exhibition conditions, packing and storage of the vegetable species [6].

Among the vegetable species more marketed in markets and free markets of São Luís/MA show of the stands out *Tabebuia avellanedeae* Lor. ex Griseb. (ipê-roxo). The bark stem in form of infused is reputed to have medicinal properties against inflammatory process, cancer, healing and microbial [6,7]

Objective

To evaluate the quality of samples of bark of the stem of *Tabebuia avellanedeae* Lor. ex Griseb. (ipê-roxo), saled in São Luís/MA. markets

Materials and Methods

- **Material**

For the accomplishment of that study the public markets were selected where happens sale of medicinal plants, of larger frequency of consumers and located in neighborhoods different from the capital from Maranhão (Mercado Central , of the Praia Grande, of João Paulo, of COHAB and of the Liberdade); being accomplished simple aleatory sampling of the banking of sale of plants for medicinal use identified in those selected markets, 15 (fifteen) sale banking. In those places, they were acquired, for purchase, samples of bark of stem of *Tabebuia avellanedeae* (ipê-roxo), in the period of September of 2000 to February of 2001. All acquired material was conditioned separately in sterile, identified plastic sack and maintained in conditions adapted for the accomplishment of the analyses.

- **Methods**

The evaluation of the samples quality of the trades of *Tabebuia avellanedeae* (ipê-roxo) it was based in macroscopic analyses and microbiological for fungus.

For the macroscopic analyses, the acquired samples were, in separate, distributed by quarteament and, soon after, analyzed by direct processes of identification, as the color characteristics, consistency, size, thickness, particularities of the drug, indications of deterioration and/or presence of strange material in the rehearsal taking. The macroscopic and morphologic characteristics were observed and analyzed based on the determinations of the specialized literature [1,7,8,9]

In the microbiological analysis for fungus, uniform fragments of the commercial samples in study were inculated in plate with half Sabourand agar Dextrose and maintained in room temperature of 5 to 7 days. After the development of the fungus colonies, the microcultive technique was accomplished in sheet, using blue lactofenol of cotton as coloring. The identification was based in the observation of the macroscopic morphology of the colonies and in the microscopic study of the vegetative organs and of frutification of the fungus cultivated by the microcultive technique [10,11,12]

Results

The commercial samples of *Tabebuia avellanedeae* (ipê-roxo) acquired in the sale banking in markets of São Luís/MA, they presented alterations of the macroscopic characteristics, evidenced by differences and variations color marcantes, size, thickness, consistency and weight; being verified quite damaged samples, with clear indications of deterioration and signs of microbial contamination in the external and internal area of the barks of the stems. Those indicative of the bad quality of the analyzed vegetable drug alterations of macroscopic characteristics, the inadequate conditions should be related from the cultivation to the storage during the commercialization.

The inoculation of uniform fragments of those commercial samples in Sabourand dextrose half agar microcultiv resulted in the growth of several colonies of fungus, being proven contamination fúngica in whole the rehearsal takings. They were identified fungus *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Absidia* ssp., *Rhizopus* ssp. and *Penicillium* ssp.; observed that *Aspergillus niger* is the present species in all the analyzed samples.

The fungal contamination of those samples should be related the inadequate conditions of crop, processing powder-crop and storage during the commercialization, representing great risk, considering that mushrooms, mainly of the gender *Aspergillus,* is responsible for the production of toxicant substances (micotoxinas), as the aflatoxinas, could cause damages to the human beings due to high toxicidad and the capacity of destruction e/ou alteration of the active beginnings; testing, like this, for the bad quality of the vegetable drug in study, the fiscalization need and control of the vegetable material marketed for medicinal ends in markets.

Conclusion

The presented results allow we verify that the commercial samples of *Tabebuia avellanedeae* (ipê-roxo) acquired in the sale banking in markets of São Luís/MA they were inappropriate for the consumption, being evidenced variations of macroscopic characteristics and contamination fungal, not identifying species of fungus allowed by the effective legislation and specialized literature.

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QC-24 A COMPARATIVE STUDY OF RABBIT PYROGEN TEST, WHOLE BLOOD CELL CULTURE AND *LIMULUS* AMOEBOCYTE LYSATE ASSAY IN PYROGEN TESTING

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Introduction

Pharmaceutical products for parenteral administration must be free of pyrogens such as lipopolysaccharides (LPS), bacteria, fungi and virus. Pyrogens are a heterogeneous group of compounds inducing fever upon intravenous injection. Besides the possibility that a pharmaceutically active substance itself or an additive is pyrogenic when applied parenterally, any product may be contaminated with pyrogenic substances from microbial sources such as endotoxin. Sterile pharmaceuticals are normally tested for pyrogenic contamination by the rabbit pyrogen test or *Limulus* amoebocyte lysate (LAL) test. The rabbit pyrogen test is based on the fact that there is a temperature increase in animals following intravenous injection of pyrogen. Rabbits have a sensitivity for pyrogens comparable to humans and the rabbit pyrogens test recognises all kinds of pyrogens, but major disadvantages of this test are that it is costly and uses animals. The LAL test is very sensitive in the detection of LPS but it does not detect pyrogens other than LPS and often poses problem when applied to solutions with a high protein content such as blood preparations.

Goals

In order to overcome some of the shortcomings of the rabbit test and LAL test and establish an *in vitro* alternative to animal testing, monitoring the production of interleukin-6 (IL-6) by the human whole blood in response to pyrogens, were standardized for the evaluation of commercial batches of pharmaceuticals intended for parenteral administration.

Methodologies

Rabbit pyrogen test, *Limulus* amoebocyte lysate (gel cloth and chromogenic tests) and cell culture of human whole blood, with pyrogen-stimulated interleukin-6 (measured by immunoassay) as the readout.

Results and Conclusions

The results obtained with several batches of different commercial products tested by LAL semi-quantitative gel-cloth and quantitative chromogenic methods are shown in Table 1. In most of the results, there was good correlation between the gel cloth and chromogenic end point procedures.

Table 1. Results of chromogenic and gel cloth *Limulus* amoebocyte lysate test on pharmaceutical products

	LAL Test	
PRODUCTS	Chromogenic IU/ ml	Gel Cloth IU/ ml
Diclofenac sodium	<0.06	<0.06
Ranitidine	1.37	1.2 – 2.4
Heparin	<0.06	<0.06
Erythropoietin	16.13	7.68 – 15.36
Furosemide	<0.06	<0.06
Methylprednisolone	146	245.7 – 491.5
Insulin	<0.06	<0.06
Vancomycin	3.2	2.4-4.8
Dexamethasone	<0.06	<0.06
Oxacillin	<0.06	<0.06
Gentamicin	<0.06	<0.06

Only batches of products with endotoxin concentration higher than 13.81 IU/ml induced positive responses in the rabbit pyrogen test. Some of the samples were tested using the IL-6 responses of human whole blood monocytes. The standards were the 2nd International Standard for bacterial endotoxins (WHO 94/580) and the 1st International Standard for Interleukin-6 (WHO 89/548). A comparison of the endotoxin content of different pharmaceutical products as detected in a human whole blood assay, a LAL test and a rabbit pyrogen test is shown in Table 2.

Table 2. Detection of pyrogens by human whole blood assay, LAL test and rabbit pyrogen test.

PRODUCTS	Methods Whole Blood IU/ ml	LAL IU/ ml	Rabbit Test
Amikacin	<0.04	<0.06	Pass
Erythropoietin	0.76	0.48-0.96	Pass
Heparin	<0.04	<0.06	Pass
Human Serum Albumin	2.4	4.8-9.6	Pass
hGH	12.4	15.84-31.68	Pass
Metoclopramide	<0.04	<0.06	Pass
Furosemide	<0.04	<0.06	Pass

Wherever possible the pyrogens test in rabbits is being replaced. The *Limulus* amoebocyte lysate is an alternative that become more sensitive, but still has some variability and give false-results. The cell culture methods are a possible alternative that can contribute for the quality control of pharmaceutical preparations with higher degree of specificity.

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QC-25 PHYSICOCHEMICAL CHARACTERIZATION AND BIOLOGICAL EVALUATION OF RECOMBINANT HUMAN ERYTHROPOIETIN

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Introduction

Erythropoietin (EPO) is a glycoprotein hormone, synthesized mainly in the kidney, which stimulates erythropoiesis through actions on erythroid progenitor cells. Human EPO consists of a single 165 amino acid polypeptide chain which is heavily glycosylated, corresponding to 40% of the molecular mass of approximately 30 kDa. The bioactivity of EPO is directly related to the number of sialic acid residues that terminate the majority of the carbohydrate chains attached to the protein and give the molecule its low (acidic) isoelectric point. Recombinant DNA-derived human erythropoietin (rhEPO) has been produced as a glycosylated protein in Chinese hamster ovary (CHO) cell lines, with the EPO gene cloned into that cell line. The purified recombinant protein is now widely available as a therapeutic, and is used in the treatment of anaemia in patients who lack the normal source of EPO, the human kidney.

Goals

Assessment of the biological potency, identification and purity evaluation of pharmaceutical preparations of rhEPO.

Material and Methods

Bioassay in normocythaemic mice 8 week-old with the European Pharmacopoeia Biological Reference Preparation (Ph. Eur. BRP) for rhEPO as a standard. The animals were fully randomised and each received a single subcutaneous injection of 10, 30 and 90 IU/0.5 ml. Blood was collected on day 5 and the reticulocytes counted. Non-reduced sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with immunoblotting using a specific anti-EPO polyclonal antibody. Isoelectric focusing (IEF) combined with immunoblotting. IEF blots with the biotinylated lectins from *Triticum vulgaris, Lycopersicon esculentum, Canavalia ensiformis* (Concanavalin A), *Maackia amurensis,* and *Erythrina cristagalli.* Size-exclusion high-pressure liquid chromatography.

Results and Discussion

Using the Ph. Eur. BRP for rhEPO as a reference, purity and molecular weight were assessed by SDS-PAGE and immunoblotting giving a single broad staining band with an approximate molecular weight of 34 kDa. The isoforms were revealed by isoelectric focusing (IEF) and visualized also by immunoblotting. Extensive heterogeneity was shown by the different preparations consisting of 6 or more isoforms distributed over the pI range 4.0 – 5.0, as shown in Figure 1.

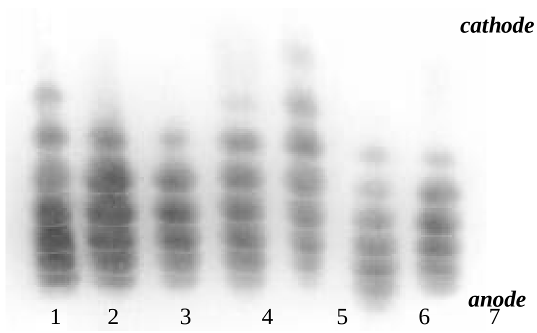

Figure 1. Isoelectric focusing profiles of rhEPO in pharmaceutical products after immunoblotting developed with alkaline phosphatase. Lane 1: Ph. Eur. BRP for rhEPO; Lanes 2 to 7 rhEPO samples A to F.

The degree of sialylation of glycoprotein hormones has been shown to be directly related to their biological potency by influencing pharmacokinetic parameters. Glycosylation variants (glycoforms) were analysed by probing the IEF blots with the biotinylated lectins specific for terminal sugars on the N-linked glycan chains and may provide detailed information, for example, on the degree and type of sialylation or the presence of free galactose or mannose groups and can therefore provide a check on the consistency of manufacture. Dimers and higher molecular mass-related substances in several commercial batches of rhEPO were comprised from 1.4 to 13.6% of the material. This could have important consequences therapeutically since changes in the specific activity of the material could affect its in vivo biopotency. Biological potency was assessed in the i*n vivo* normocythaemic mouse bioassay with reticulocytes counting carried out by microscopic examination of supravitally-stained blood films or after selective haemolysis, and by an automated method using flow cytometric analysis.

The test is labour-intensive and lacks precision and accuracy, giving a coefficient of variation (CV) of 20.4% for the microscopic method. The precision and correlation between different methods was evaluated. For example, the effects of either a single or a daily injection of rhEPO on the time course were evaluated in terms of highest biological response and lowest coefficient of variation establishing the standardized bioassay protocol. The potencies of the manufacturers' preparations calculated by a parallel-line statistical method varied between 85.11 to 106.17% of the stated potency. The precision index, expressed as the weight for the bioassay, for all the reticulocytes counting procedures varied between 358 and 675, from a combination of three independent bioassays. The samples were also used to evaluate other parameters such as haemoglobin content, haematocrit and total erythrocyte population, all of which showed an increase in all assays.

Conclusion

Recombinant biological therapeutics produced by the biotechnology industry are generally highly complex molecules that should be assessed using a range of well-validated analytical techniques in order to ensure a high degree of quality control of their biological and physicochemical properties.

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